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**Biodiesel and bioethanol production from the terrestrial algae and
their by-products**

**Report submitted to Karnataka State Council for Science and Technology in
partial fulfillment for 40th Student Projects Programme (Biofuels)**



By

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2017.

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ACKNOWLEDGEMENT

The realization of the piece of work wouldn't have been possible unless the contribution of many people, who rendered their kind help in one or the other way and so, comes the time to look back on the path the traversed during this endeavor and to remember the face and spirits behind the action with a sense of gratitude.

I avail this opportunity to express my deep sense of gratitude and indebted to my external advisor **Dr. Mohan Chavan**, Assistant Professor, Department of Plant Biochemistry, Agricultural Biotechnology block, Agriculture College Hassan sub campus of University of Agricultural Sciences Bengaluru, for his learned counsel encouragement, valuable guidance, sustained interest untiring help, caring and constructive criticism throughout my research work. His guidance helped me in all the time of research, to overcome a lot of difficulties and writing of this thesis.

I am privileged to express my deep sense of gratitude and profound regards to **Mrs. Arti Karosiya Chavan**, Ph.D. Scholar, UHS, Bagalkot, for her esteem guidance and noble supervision throughout my research work.

I shall be grateful to **Dr. L. Manjunath**, Dean and all the teaching staff of Agriculture College Hassan, for their co-operation and encouragement during this course of study and I am thankful to **Chandarshekar H. C**, lab assistant, Agricultural Biotechnology Block, Agriculture College Hassan, for his keen interest in my research work sympathetic ear and making me comfortable in the lab.

With all respect I wish to express my heartfelt gratitude to **KSCST** and **Karnataka State Biofuel Development Board** for project funding during this project work.

My heartfelt to my beloved parents, grandparents, sister and my family members for their invaluable sacrifice, keen enthusiasm, cordial encouragement and assistance bestowed on me throughout my educational carrier which made me what I am now.

I would like to express my special gratitude to my best friend **Miss. Mamatha P** for shaping my personality, constant encouragement and moral support. Her guidance helped me to overcome lot of difficulties during writing of this report.

Finally I offer my thanks to any other source of contribution that might have inadvertently have been left out.

DECLARATION

I, **Manoj B. S.** student of **M.Sc. Agricultural Biochemistry**, hereby declare that the work conducted and the evaluation embodied in the report, “**Biodiesel and bioethanol production from the terrestrial algae and their by-products**” was performed on my own, under the supervision and guidance of **Dr. Mohan chavan**, Department of Biotechnology, Agricultural College, Hassan.

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Date: 18/07/2017

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LIST OF ABBREVIATIONS

AEPS	Aqueous extract of piper sarmentosum
APS	Ammonium persulfate
BBM	Bold basal medium
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CAT	Catalase
C.D.	Critical difference
COD	Chemical oxygen demand
DHA	Docosahexaenoic acid
DNS	3, 5-dinitrosalicylic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FCR	Folin-Ciocalteu reagent
Fe-EDTA	Iron-Ethylene diamine tetra acetate
NBT	Nitroblue tetrazolium
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
pH	Potential of hydrogen
POD	Peroxidase
PPM	Parts per million
PVP	Polyvinylpyrrolidone
R.O.	Reverse osmosis
ROS	Reactive oxygen species
RPM	Revolution per minute
RSA	Radical scavenging activity
S.D.	Standard deviation
SDS	Sodium dodecyl sulphite
S.E.	Standard error
SEM	Scanning electronic microscope
SOD	Superoxide Dismutase
TDS	Total dissolved solid
TEMED	N,N,N',N'- tetramethylethylenediamine
TRS	Total reducing sugar
TSS	Total soluble sugar

Abstract

Algae are photosynthetic organisms, present in single to multicellular form. Algae generally found in soggy or wet places and all types of water bodies. Algae-based biofuels are technically and economically viable, cost competitive and mitigate atmospheric CO₂. Extracted oil after transesterification and few alkaline wash gives biodiesel and it can be used by blending with petro diesel. Biodiesel from the microalgae solves the major drawbacks associated with oil crops and lignocellulotic-based biofuels. Biodiesel has many advantages like biodegradable, nontoxic and it has lower emissions of greenhouse gases. In this study around 62 terrestrial algae were collected from the fresh water bodies of Western Ghats surrounding regions. These collected algae were identified based on microscopic and biochemical characteristics. Out of these only 7 samples of high oil content and biomass yielding samples were identified and used for multiplication and fermentation study. Maximum oil content found was 43±0.75% and minimum was 2.15±0.5 %. Multiplication of algae was done by closed photobioreactor system with sparger developed for continuous agitation and also performed in open pond production system. All collected and characterized seven algae samples are showed good response to various water treatments. Algae are harvested by filtration and centrifugation methods. The left water can be used for multiple purposes like culturing same algae, irrigation, dust control, construction purpose etc. In addition it was documented the biochemical composition of the terrestrial algae resulting in higher oil content. The study shows convert algal oil into biodiesel by transesterification process and producing bioethanol by fermentation setup using algal defatted residues after acid hydrolysis with 2.5N HCl and neutralized with Na₂CO₃ used as a substrate for fermentation and 5% of *Saccharomyces cerevisiae* inoculum. In present study maximum 61.76±1.12% and minimum 57.51±0.99% alcohol was obtained. During the process of fermentation, sugars from defatted algal residue are broken down and converted into ethanol and carbon dioxide by *Saccharomyces cerevisiae*. Algae are 3rd generation biofuels doesn't compete for land and space with other agricultural crops, it also survive in alkaline water.

Key word: Biofuel, Algae biodiesel, Bioethanol, Algae, *Rhizoclonium Sp*, *Saccharomyces cerevisiae*.

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INTRODUCTION

Algae are photosynthetic organisms present in single to multicellular form. They are generally found in soggy or wet places and all types of water bodies hence algae are common in aquatic and terrestrial environments (**Wagner, 2007**). Microalgae are a group of mostly photoautotrophic microorganisms that includes both prokaryotic and eukaryotic species. These organisms can photosynthetically convert CO₂ and minerals to biomass, but some species also grow heterotrophically. Prokaryotic microalgae are cyanobacteria (blue-green algae) and eukaryotic microalgae include the nine phyla *Glaucophyta*, *Chlorophyta*, *Chlorarachniophyta*, *Euglenophyta*, *Rhodophyta*, *Cryptophyta*, *Heterokontophyta*, *Haptophyta* and *Dinophyta* (**Duong et al., 2012**).

Algae biomass is proved to be an effective raw material for many useful products such as fuel oil, fertilizers, protein and other substances. The advantage is that it does not compete with conventional crops and agricultural crops (**Govindarajan et al., 2010**). Microalgae are an important source for biomolecules such as proteins, lipids, vitamins, pigments and other molecules exploited for health, food and feed additives, cosmetics and for energy production. It contains wide range of biochemical compounds and antioxidant agents like: pigments including carotenoids, phenolic compounds, sulfated polysaccharides and long-chain polyunsaturated fatty acid (**Maadane et al., 2015**).

Microalgae which contain at least 30% lipids in the microalgae cell have possibility to be used to convert biofuel. Through photosynthesis, microalgae can absorb large amounts of CO₂ to produce sugars and oxygen. The photosynthesis reaction equation as follow:



The sugars produced can convert to lipids, proteins and carbohydrates, which are as the materials can be converted to the biofuel (**Field et al., 1998**).

Microalgae are the photosynthetic microorganisms, which are attracting huge interest from researchers, government, and local and international entrepreneurs. Recently, the usage of liquid biofuels such as biodiesel, bioethanol, and jet fuel has increased immensely especially in the transport industry (**Tabatabaei et al., 2011**). Compared to fossil diesel, biodiesel has many advantages such as it is biodegradable, nontoxic and it has lower emissions of greenhouse gases (**Demirbas, 2008**).

Microalgae biofuel is one of the major renewable energy sources for sustainable development, with potential to replace the fossil-based fuels. Microalgae biofuel was devoid of the major drawbacks associated with oil crops and lignocelluloses-based biofuels (**Medipally et**

al., 2015). Microalgae can able to cultivate in seawater, brackish water, or waste water and these microalgae are not compete for arable land with conventional agriculture species (**Wijffels and Barbosa, 2010**). Microorganisms contain a wide variety of carbohydrates, both monomers and polymers. Different classes of algae produce specific types of polysaccharides, green algae produce starch as an energy store, consisting of both amylose and amylopectin. Microalgae can able to adapt new environments through the de novo synthesis and recycling of fatty acids to maintain the membrane characteristics (**Peter et al., 2010**).

Algae are almost ideal as organisms for developing the highly productive and robust crop strains that are essential for economically viable biofuel production. The search for these strains can exploit the vast diversity of algae, ranging from giant multicellular kelps to single-celled microalgae (**Georgianna and Mayfield, 2012**). Microalgae cultivation is the first production step in the energy production system. In this experiments microalgae cultivation was done by commonly using methods like: Open pond system, Close photo bioreactors system and Heterotrophic production system (**Demirbas, 2008 and Padmanabhan, 2012**)

Biomass energy sources have three generations development steps. The first generation biofuel feedstocks derived from food crops, such as sugarcane, corn and wheat. Rice straw and switch grass as sorts of non-food raw materials become the second generation biofuel feedstocks. The third generation biofuel feedstock is algae (especially microalgae). Algae will probably play an increasing role in the sustainable energy use field in the near future (**Patil and Tran, 2008**).

The first-generation biofuel produced from grain, sugar, oilseed crops, maize kernels, soybean or other starchy crops. It raised the cost of certain necessary staple food crops and food stuffs. Agricultural crops use towards energy generation cause competition for food (**Shraddha et al., 2016**). However second-generation biofuel are produced from lignocelluloses biomass that is not edible and oil produced from food waste, farm slurry, chicken litter, and non-edible oil tree species. It requires most sophisticated processing production equipment, more investment per unit of production and larger-scale facilities to confine and curtail capital cost scale economies. It also caused increased in the removal of forested lands for growing fuel crops and trees species (**Nigam and Singh, 2011**).

The above limitations favor the search of non-competitive third generation biofuel such as algae for the production of biodiesel and bioethanol. Microalgae can be considered as major feedstock for biodiesel production because (1) Algae as 200 times more productive per hectare than a land-based crop, and reduce pressures on land use (**Shraddha et al., 2016**), some species growth can be doubled in a few hours; (2) some of the algae species can accumulate large amounts of triacylglycerides, and (3) it not required high quality agricultural land to grow the biomass (**Scott et al., 2010**).

Algal oil can be converted into biodiesel by Transesterification method. Transesterification is a reaction between fatty acid or ester molecule and alcohol, where organic R group of ester and R' group of alcohol is exchanged. Algal lipids have viscosity higher than diesel; transesterification reduces the viscosity of algae lipids. Ethanol, Amyl alcohol Methanol, Propanol, Butanol are used for this reaction. Methanol is widely used because its low cost. This reaction is carried out in presence of an acid or base catalyst. Acids can catalyses reaction but rate of conversation is extremely slow. Base catalyst mediated transesterification is 4000 times faster than acid transesterification. The base catalyst have higher reaction rate for transesterification of TAG (**Huang et al., 2010**).

Microalgal biodiesel is technically feasible. It is the only renewable biodiesel that can potentially and methodically displace liquid fuels obtained from petroleum. Economics of producing microalgal biodiesel need to improvise substantially to make it competitive with petro diesel, therefore the level of improvement for increasing the yield of microalgal biomass production and biodiesel extraction is necessary. Producing low-cost microalgal biodiesel requires primarily improvements in open pond for increasing biomass of microalgae will further reduce the cost of production of the microalgal biomass required for making biodiesel. In the second place, the research can provide some evidence as to whether or not it is efficient and sustainable at present to produce biodiesel from microalgae cultivated in wastewater. If the answer is yes, then it will be practical and reasonable to make full use of this kind of feedstock. Byproducts obtained from the microalgae during lipid extraction (algae residue) can be used as substrate for bioethanol production by fermentation of residual biomass. And byproduct obtained during biodiesel production by transesterification such as glycerin can also use as one product. In view of the above mentioned facts the present study has been designed to enhanced the production of biodiesel and bioethanol from the terrestrial algae and utilize their byproducts economically.

Keeping in view the above information, the present study has been planned with the following objectives:

- To investigate the multiplication and biochemical characterization of the high oil and biomass yielding terrestrial microalgae.
- To estimate the biodiesel production from algal lipid extracts.
- To evaluate standardization of ethanol production by fermenting defatted algal residue.
- To determine the effect of algal growth on water treatment.

REVIEW OF LITERATURE

2.1 Algae as a biofuel:

Scott *et al.* (2010) studied the yield of biodiesel from algae. According to their research productivity and lipid content are inversely correlated, and stress conditions such as deprivation of nitrogen or phosphate, which limit cell growth and increase lipid content. The lipid content of *C. vulgaris* grown under nutrient-sufficient conditions was between 14% and 30% of dry weight, values of up to 70% of dry biomass have been reported under nutrient deficiency. Excess carbon from photosynthesis is channeled into storage molecules, such as TAGs or starch and protein content may be reduced. They establishing the feasibility of algal biofuel production regardless of energy input were an important step in providing the platform for optimization, and also for establishing promising lines for future research.

Munir *et al.* (2013) reported that the process of extraction and purification of oil from algae is significant challenge in production of both microalgae by-products and biofuel. According to them without proper harvests techniques, oil extraction from algae was relatively energy-intensive and expensive. And they focused on different harvests and extraction processes of algae for biodiesel production.

Abishek *et al.* (2014) commercialized biodiesel, according to them biodiesel is generally blended with petro diesel, but it can also be used in pure form also. They reported that biodiesel was a sustainable fuel, as it was available throughout the year and can run any engine and it will meet the demands of the future generation to come. According to them biodiesel provides more environmental benefits, and being a renewable resource. Economics of producing microalgal biodiesel need to improvise substantially to make it competitive with petro diesel, but the level of improvement necessary appears to be possible. Producing low-cost microalgal biodiesel requires primarily improvements to algal biology through genetic and metabolic engineering. They explained the use of the bio-refinery concept and advances in photobioreactor engineering will further reduce the cost of production.

Behera *et al.* (2015) reported that the algal biomass can be implemented for economic conversion processes producing different biofuels such as biodiesel, bioethanol, biogas, biohydrogen, and other valuable co-products. And they found that the advance developments in algal biomass for improved biofuel production have been explored. They explained current research and technology based on the third generation biofuels derived from algal biomass have been considered as the best alternative bioresource that avoids the disadvantages of first and second generation biofuels. They discussed about the importance of the algal cell contents,

various strategies for product formation through various conversion technologies, and its future scope as an energy security.

Saber *et al.* (2016) reviewed the processes for bio-oil production from biomass: pyrolysis and hydrothermal liquefaction. One of the main challenges in bio-oil application was the poor quality of the bio-oil. It has undesirable properties such as high oxygen content and acidity which requires upgrading of the bio-oil for fuel application. They mainly reviewed on bio-oil production and upgrading techniques from algae (micro- and macro-algae). The chemistry and characteristics of bio-oil as well as recommendations for future works are also explained by them.

2.2 Techniques for growing and identification of microalgae:

Chen (1996) studied the formation of algal products from various cultivation systems, with emphasis on the use of heterotrophic techniques. The potential employment of heterotrophic high cell density strategies for commercial production was studied. They explained that the heterotrophic techniques can easily multiply the cells of microalgae within the short period of time. The lipid content in the heterotrophic cells could be four times higher than that of autotrophic cells under similar conditions.

Borowitzka (1999) reported the main problem faced during the commercialization of new microalgae and microalgal products in closed culture systems. It was a capital intensive. They avoided this problem by grown the algae heterotrophically and explained the production of microalgae for aquaculture in smaller scale. They also studied the tubular photobioreactor system, developed to grown microalgae reliably outdoors at high cell densities in semi-continuous culture

Norsker *et al.* (2011) investigated the microalgal biofuel production worldwide. Microalgal biomass production costs were calculated for 3 different micro algal production systems operating at commercial scale: open ponds, horizontal tubular photobioreactors and flat panel photobioreactors. The important cost factors are irradiation conditions, mixing and photosynthetic efficiency of systems, medium- and costs. At this cost level microalgae become a promising feedstock for biodiesel and bulk chemicals.

Duong *et al.* (2012) reported different species of microalgae which function best at different aquatic, geographical and climatic conditions. In addition, other value added products being considered for commercial production which necessitates the selection of the suitable algae strains for multiple-product algae biorefineries. A combination of conventional and modern techniques is likely to be the most efficient route from isolation to large-scale cultivation. They also reviewed the practical issues of several simple and robust methods for microalgae isolation and selection for traits that maybe most relevant for commercial biodiesel production. According

to them biodiesel production from microalgae is being widely developed at different scales as a potential source of renewable energy with both economic and environmental benefits.

Ponnuswamy *et al.* (2013) utilized scanning electron microscopic to examine morphology of *Chlorella vulgaris*. They observed algal cell under light microscope for their morphological features and other cellular details, the cells were further studied using scanning electron microscope (SEM) method. The sample was screened by SEM (Scanning Electron Microscopy) for their absolute morphological studies. The basic steps for SEM sample preparations are fixing it with buffered aldehyde, post fixing it in Osmium tetroxide, dehydrating it in ethanol, drying it with air dryer, mounting it on a specimen stub, coating it with Carbon and examining under the SEM.

Medipally *et al.* (2015) reported the algae-based biofuels are technically and economically viable and viability of microalgae biodiesel production can be achieved by designing advanced photo bioreactors, developing low cost technologies for biomass harvesting, drying, and oil extraction. Commercial production can also be accomplished by new emerging technologies such as algal-bacterial interactions for enhancement of microalgae growth and lipid production are also explored. They focus mainly on the problems encountered in the commercial production of microalgae biofuels and the possible techniques to overcome these difficulties.

2.3 Harvesting techniques of microalgae:

Mohn (1980) harvested algal biomass using centrifugation. This research focused on suitability of algal strains, cost and energy use of Mohn's findings indicating the possible harvesting methods, effectiveness, energy requirements and reliability of several harvesting methods. Mohn's results suggest filtration provides the best harvesting strategy in terms of high concentration of solids with low energy requirements.

Golueke and Oswald (1965) investigated various methods of dewatering algae for provide a biomass with sufficiently low moisture content. One of the methods they looked was centrifugation. They tested for dewatering of algae sample by using centrifuge method and proved that centrifugation techniques extremely effective in removing maximum 79% of water from the biomass of microalgae.

Heasman (2000) explained that centrifugation recovery (CR) is preferred for harvesting of high value metabolites and extended shelf-life concentrates for hatcheries and nurseries in aquaculture. The process is rapid and energy intensive; biomass recovery depends on the settling characteristics of the cells, slurry residence time in the centrifuge, and settling depth. The disadvantages of the process are high energy costs and potentially higher maintenance

requirements due to freely moving parts. Harvesting efficiency of >95%, and increase in slurry concentration by up to 150 times for 15% total suspended solids are technically feasible.

Liu et al. (2013) explained the methods of harvesting microalgae by mechanical, electrical, biological and chemical based methods. They used flocculation method which induced by decreasing pH value of growth medium was developed for harvesting freshwater microalgae. The flocculation efficiencies were as high as 90% for *Chlorococcum nivale*, *Chlorococcum ellipsoideum* and *Scenedesmus sp.* with high biomass concentrations (>1g/L). According to them the optimum flocculation efficiency was achieved at pH 4. The pH value of each sample was gradually adjusted by addition of 1M HNO₃. They also showed the potential to overcome the hurdle of harvested microalgae to promote full-scale application to biofuels from microalgae.

Milledge and Heaven (2013) studied harvesting of microalgae by a number of methods: sedimentation, flocculation, flotation, centrifugation and filtration or a combination of any of these. They also explained a various methods of harvesting and dewatering of microalgae for the production of biofuel.

Show et al. (2017) observed that centrifugation as a popular method for aggregating metabolites due to its fast operation and high efficiency. They also explained centrifugation method is an energy-consuming due to settling characteristics of the cells, the slurry residence time in the centrifuge and the settling depth. The weaknesses of this method include high energy consumption and the requirement for maintenance due to free moving parts. They explained several harvesting methods and its advantages and disadvantages. Therefore, making a proper choice of harvesting method will have an effect on the economic outcome of the investment. The type of harvesting technique used relies solely on the characteristics of the microalgae, for instance the density, size, and desired output. They categorized the two main method of harvesting microalgae are bulk harvesting and thickening.

2.4 Algal oil converted in to biodiesel production by transesterification:

Huang et al. (2010) observed that algal lipids have higher viscosity than diesel; transesterification reduces the original viscosity of algae lipids. Ethanol, Amyl alcohol Methanol, Propanol, Butanol are used for this reaction. Methanol is widely used because of its low cost. Base catalyst mediated transesterification is 4000 times faster than acid transesterification. The base catalyst have higher reaction rate for transesterification of TAG therefore they are used in commercial production of biodiesel.

Musa (2014) reviewed the nature of alcohol and alcohol to oil molar ratio plays an important role for the method of biodiesel production. In their paper found that different alcohols commonly used for the production of biodiesel fuel with more emphasis on methanol and

ethanol. Further the different alcohol to oil molar ratios used for the production of biodiesel have been extensively discussed and reported. Further the effects of alcohol to molar ratios on biodiesel refining process and its physicochemical properties were also investigated. They found that methanol does not form azeotrope; hence its recovery was simple compared to ethanol. Although ethanol is more expensive than methanol but biodiesel production involving ethanol was completely bio base. Most of the researchers recommend a 6:1 M ratio for methanol and a 9:1 M ratio for ethanol. They suggested to take care for determine empirically ideal molar ratios to employ otherwise excess methanol will result in severe difficulty in biodiesel refining process. They found that the presence of alcohol affects the quality of biodiesel fuel by lowering its viscosity and density values, and flash point.

2.5 Bioethanol production by fermentation of algal residues:

Govindarajan *et al.* (2010) reviewed algae as autotrophic organisms that produce complex organic compounds from simple inorganic molecules such as carbon dioxide using energy from light or inorganic chemical reactions. These complex organics include a substantial amount of triacylglycerol which can be readily converted into biodiesel by the well-known transesterification process. In addition, the residual algal biomass which results after the extraction of the triacylglycerol can also be utilized to produce ethanol, value-added chemicals, and animal feed.

Harun and Danquah (2011) studied a central composite design technique for optimizing acid pre-treatment conditions. The highest bioethanol concentration obtained was 7.20 g/L and this was achieved when the pre-treatment step was performed with 15 g/L of microalgae at 140°C using 1% (v/v) of sulphuric acid for 30 min. In terms of ethanol yield, ~52 wt% (g ethanol/g microalgae) maximum was obtained using 10 g/L of microalgae and 3% (v/v) of sulphuric acid under 160°C for 15 min.

Harun *et al.* (2014) studied the total carbohydrates present in the biomass could be made available for bioethanol production under optimal saccharification and microbial fermentation conditions. They found carbohydrate constitutes up to 32% of the dry weight of *C. infusum* biomass with the major fermentable sugar component being glucose (15.2%), followed by xylose (9.5%), mannose (4.9%), and galactose (2.9). They used *Saccharomyces cerevisiae* in the microbial fermentation process for bioethanol production and cultured yeast in YDP. The yeast was harvested after 24 h and washed to eliminate the sugars then transferred into 500mL Erlenmeyer flask containing 100mL of the sugar-containing liquid medium obtained after the hydrolysis process. The flasks were tightly sealed and nitrogen gas was bubbled through to create an oxygen-free environment for bioethanol production. The flasks were incubated at 30°C under 200 RPM shaking. The pH was maintained at 7 by adding 1M NaOH solution. The fermentation continued for 50 h and samples for analysis were taken after every 4 h.

Imran et al. (2016) converted algal biomass to algae juice through a specific pretreatment method. The resulted algae juice was used as a substrate in fermentation process. Highest yield of bioethanol (50mM/mL) was detected when *Brettanomyces custersainus*, *Saccharomyces cerevisiae*, and *Pichia stipitis* were used in combinations for fermentation process as compared to their individual fermentation. Individually *B. custersainus* showed higher yield of fermentative ethanol as compared to *S. cerevisiae* and *P. stipites*. The results indicated the influence of different factors on the growth rate and carbohydrates productivity of *M. aeruginosa* and its feasibility as a feedstock for fermentative ethanol production.

Yuko et al. (2016) reported *Euglena gracilis* as a eukaryotic, unicellular phyto flagellate that has been widely studied in basic science and applied science. Under dark, anaerobic conditions, the cells of *E. gracilis* produce a wax ester that can be converted into biofuel. Here, they demonstrate that under dark, anaerobic conditions, *E. gracilis* excretes organic acids, such as succinate and lactate, which are bulk chemicals used in the production of bioplastics. The levels of succinate were altered by changes in the medium and temperature during dark, anaerobic incubation. Succinate production was enhanced when cells were incubated in CM medium in the presence of NaHCO₃. Excretion of lactate was minimal in the absence of external carbon sources, but lactate was produced in the presence of glucose during dark, anaerobic incubation. *E. gracilis* predominantly produced L-lactate; however, the percentage of D-lactate increased to 28.4% in CM medium at 30°C. They used a commercial strain of *E. gracilis* for succinate production and found that nitrogen-starved cells, incubated under dark, anaerobic conditions, produced 869.6 mg/L succinate over a 3-day incubation period, which was 70-fold higher than the amount produced by nitrogen-replete cells.

2.6 Biochemical composition of Microalgae:

Peter et al. (2010) found that microorganisms contain a wide variety of carbohydrates. Different classes of algae produce specific types of polysaccharides. Green algae produce starch as an energy store, consisting of both amylose and amylopectin, similar to higher plants. The green algae *Tetraselmis suecica*, accumulates 11 and 47% of its dry weight as starch in nutrient replete and deplete conditions respectively. Proteins also have both structural and metabolic functions. As enzymes, they are the prime catalysts for cell metabolism and so facilitate growth. The structural cell wall of *Chlamydomonas reinhardtii* consists primarily cross-linked hydroxy-proline-rich glycoproteins.

Adarme et al. (2012) states that Heterotrophic microalgae have been used for the production of omega-3 fatty acids, in particular DHA. In a bio refinery concept, omega-3 fatty acids can be separated from microalgal lipids which would be widely used for biodiesel production. Biomass can be used as valuable protein-rich animal feed which could free up arable land for food

production. If microalgae cultured at a large scale would address three major areas of importance: human health, transportable energy and food security.

2.7 Algae use as bioremediation of waste water:

Kshirsagar (2013) studied the growth rate of *Chlorella vulgaris* and *Scenedesmus quadricauda* in the wastewater which increases, the rate of reduction of different pollutants or nutrients. *Chlorella vulgaris* showed the best removal capacity of nitrate and COD while *Scenedesmus quadricauda* showed best result for BOD and phosphate reduction. Unicellular green algae such as *Chlorella* and *Scenedesmus* have been widely used in wastewater treatment as they have fast growth rates and high nutrient removal capabilities. According to them *Chlorella vulgaris* and *Scenedesmus quadricauda* may be considered efficient nutrient removers and also improves water quality.

Sharma and Khan (2013) they made an investigation effort to phycoremediate the Indian Agricultural Research Institute, Pusa's sewage wastewater with different microalgae viz. *Chlorella minutissima*, *Scenedesmus spp.* & BGA (*Nostoc*) and their consortium. Results showed that these algae were very effective in reduction of BOD, COD, NO_3^- , NH_4^+ , PO_4^{3-} and TDS in sewage wastewater. After 20 days the maximum biomass was observed in *Scenedesmus spp.* and *Chlorella minutissima* while percentage of nitrogen and phosphorus was highest in *Chlorella minutissima*. The results of this study suggest that growing algae in nutrient-rich sewage wastewater offers a new option of applying algae to manage the nutrient load and after phytoremediation the biomass itself can be utilized for manure application in agriculture, serving the dual roles of nutrient reduction and valuable manure feedstock production.

Sethupathy et al. (2015) collected sewage waste water from Indira Institute of Engineering and Technology, located at Chennai, India. They treated the waste water with three micro-algal strains which were *Chlorella sp.*, *Scenedesmus sp.*, and *Nostoc* in waste stabilization pond. The Phycoremediation method is alternative methodology for treating waste water at cheaper cost. They saw maximum reduction of BOD, COD, TDS, PO_4 , NH_4^+ , in *Chlorella* treatment. *Nostoc* gave lower reduction of physicochemical parameters compared to other two micro algal strains. *Scenedesmus* algae produced more biomass which can be used for manure production. This work suggested that algae thrive in nutrient-rich waters like municipal waste waters (sewage) at the same time purifying these wastes while producing a biomass suitable for bio-fuels and manure production.

MATERIALS AND METHODS

The materials and methods used in the present study entitled “**Biodiesel and bioethanol production from the terrestrial microalgae and their by-products**” are as follows:

Place of work: The major work has been carried at Agriculture Biotechnology block (Agriculture College Hassan), University of Agricultural Sciences, Bengaluru, Karnataka

3.1 Materials:

Non-consumables and instruments:

Sl.No.	Name of instruments	Manufacture company name
01	Autoclave	KETAN SA111
02	Centrifuge machine	EPPENDORF 5418R and REMI C-30BL
03	Cyclo mixer	REMI CM-101
04	Digital pH meter	ELICO LI615
05	Distillation unit	BIO-AGE
06	Dry bath	HLC BIOTECH
07	Electrophoresis apparatus	LIFE TECHNOLOGIES MINI GEL TANK
08	Freezer	VESTFROST
09	Hot air oven	LABLINE
10	Ice machine	SIMAG SMN-75
11	Magnetic stirrer	REMI 1MLH
12	Microscope	LABOMED CXL PLUS
13	Scanning electronic microscope	QUANTA 200
14	Soxhlet apparatus	RAGHA LAB SYSTEMS
15	Spectrophotometer	CECIL CE 7400
16	TDS meter	HM DIGITAL
17	Hybridization oven	MS MAJOR SCIENCE
18	Incubating orbital shaker	SYMBIONT TECHNOLOGIES
19	Water bath	REMI RSB-12
20	Weighing machine	SARTORIUS QUINTIX224-1S

3.1.1 Glassware:

Test tubes, centrifuge tubes, pipettes, volumetric flasks, measuring cylinders, funnels, burettes, glass rods, beakers, round bottom flask etc.

3.1.2 Chemicals and Reagents:

All chemicals used in the experiments were of analytical grade. Preparation of reagents has been mentioned in appendix.

3.1.3 Samples collection:

Algal samples were collected in sterile plastic bags from different water bodies in and around the villages of Western Ghats of Karnataka. Western Ghats is highly bio diversified green belt of India. In experiments different fresh water sources of Western Ghats were chosen to collect algae, the sites were river, ponds, lakes and tanks. Around 62 terrestrial algae were collected from the fresh water bodies of Western Ghats surrounding regions. Out of these only 7 algal collections were found with high oil percentage and was used for further experimental purpose. Collected algae were used for mass multiplication and was subjected to anaerobic fermentation after defats and acid hydrolysis. These 7 samples collected from various places they are,

- River bank of Happaadka, Udupi district the village lies between Latitude 13.27'15262°N and Longitude 75.04'81859°E.
- Pond of Dandiganahalli, Hassan district the village lies between Latitude 12.9735798°N and Longitude 76.270381°E.
- Pond of Hoovnalli, Hassan district the village lies between Latitude 12.99877°N and Longitude 76.25182°E.
- Tank of Agricultural College Hassan (ACH) Campus, Karekere Hassan district the village lies between Latitude 12.9772108°N and Longitude 76.2584164°E.
- Pond of Gadanahalli, Hassan district the village lies between Latitude 13.0015338°N and Longitude 76.0831914°E.
- River bank of Ikola, Kodagu district the village lies between Latitude 12.34824° N and Longitude 75.79182°E.
- River bank of Kaggodu, Kodagu district the village lies between Latitude 12.36916°N and Longitude 75.75782°E.

Table 3.1: Collection of terrestrial microalgae from different sources and locations of in and around Western Ghatts of Karnataka

Sl.No	Place	Source	Sl.No	Place	Source
01	Hoskeri (Kodagu)	River	32	Kundapura (Udupi)	River
02	Hoskeri (Kodagu)	Pond	33	Gokarna (Uttar Kannada)	River
03	Chettali (Kodagu)	Pond	34	Shrungeri (Chikamagaluru)	River
04	Chettali (Kodagu)	Open Well	35	Koppa (Chikamagaluru)	Pond
05	Maragodu (Kodagu)	River	36	Mudigere (Chikamagaluru)	Open Well
06	Maragodu (Kodagu)	Pond	37	Mudigere (Chikamagaluru)	Pond
07	Galibidu (Kodagu)	Open Well	38	Ajjampura (Chikamagaluru)	Pond
08	Ikola Holley (Kodagu)	River	39	Kaduru (Chikamagaluru)	Pond
09	Cheyandane (Kodagu)	River	40	Biruru(Chikamagaluru)	Pond
10	Nariyandada (Kodagu)	River	41	Madagada Kere (Chikamagaluru)	Pond
11	Shanmangala(Kodagu)	River	42	Dandigana Halli (Hassan)	Pond
12	Kaggodu (Kodagu)	River	43	Karekere (Hassan)	Pond
13	Kanthur Murnad (Kodagu)	River	44	Heragu (Hassan)	Pond
14	Mekeri (Kodagu)	River	45	K. Byadaralli (Hassan)	Pond
15	Kiggall (Kodagu)	River	46	Udayapura (Hassan)	Pond
16	Kattemadu (Kodagu)	River	47	Shantigram (Hassan)	Lake
17	Mudabidri (Dakshina Kannada)	River	48	Aluru (Hassan)	Pond
18	Mudabidri (Dakshina Kannada)	Pond	49	Hoovnalli (Hassan)	Pond
19	Mudabidri (Dakshina Kannada)	Open Well	50	Yaliyuru (Hassan)	Pond
20	Beltangadi (Dakshina Kannada)	River	51	Gadanahalli (Hassan)	Pond
21	Ujjire (Dakshina Kannada)	River	52	Kavalikere (Hassan)	Pond
22	Darmastala (Dakshina Kannada)	River	53	Kandli (Hassan)	Pond
23	Sanoor(Udupi)	River	54	Bhuvanalli (Hassan)	Pond
24	Happanadka (Udupi)	River	55	Malali (Hassan)	Pond
25	Mudar (Udupi)	River	56	Kundoor Mata (Hassan)	Pond
26	Shirlal (Udupi)	River	57	ACH Campus (Hassan)	Tank
27	Ajekar (Udupi)	River	58	Aladahalli (Hassan)	Pond
28	Kervashe (Udupi)	River	59	Hittaladahalli (Hassan)	Pond
29	Arbi (Udupi)	River	60	SHUATS Campus Allahabad (UP)	Pond
30	Andaru (Udupi)	River	61	Rambagh Allahabad (UP)	River
31	Karkala (Udupi)	River	62	Triveni Sangham Allahabad (UP)	River



Figure 3.1: Collection of terrestrial microalgae from in and around Western Ghats of Karnataka

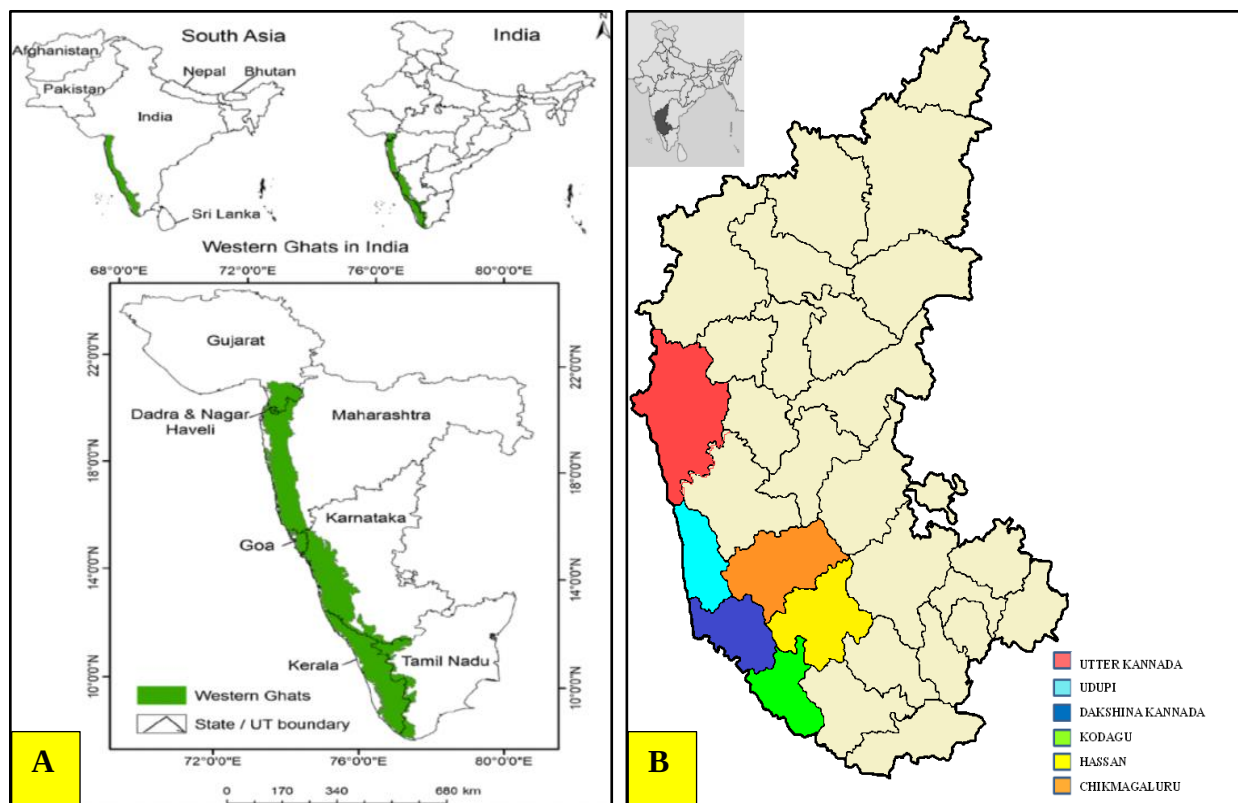


Figure 3.2: Maps indicating Western Ghats regions. A. Map showing Western Ghats states in India and B. Coloured parts of Karnataka state showing district from where algae sample were collected.

3.2 Identification of terrestrial microalgae:

Identification of microalgae was done by compound microscope and Scanning electronic microscope by the method given by *Prescott (1970)* and *Ponnuswamy et al. (2013)*.

3.2.1 Compound microscope:

Algae smear was prepared on microscopic slides by putting drop of H_2O and coverslip over it and the images were observed under compound optical Microscope Labomed CXL plus attached with camera for digital imaging was used. The images were captured at 40X and at 100X.

3.2.2 Scanning electronic microscope

The cells were further studied using scanning electron microscope (SEM) method. The sample was screened by SEM (Scanning Electron Microscopy) for their absolute morphological studies. The basic steps for SEM algae samples were dehydrated with ethanol, drying it with air

dryer, mounting it on a specimen stub, coating it with Carbon and examining under the HRSEM (Quanta FEG 200). The topography of green micro algae was analyzed.



Figure 3.3: Performing Scanning Electronic Microscope at IISC, Bangalore.

3.3 Multiplication of terrestrial microalgae:

The multiplication of terrestrial microalgae was done by the method described by **Brennan and Owende (2010)**; **Surendhiran and Vijay (2012)** and **Guerrero *et al.* (2014)**.

The algae were brought to growth phase by serial dilution. Algae solution of 200 μ l of was inoculated in solid bold basal medium (BBM). The culture plates were incubated at 25 $\pm$ 2 $^{\circ}$ C and 16hr light condition was maintained. After 18th days of inoculation subculturing was done. During the experiment time faced many challenges for isolation and growth of microalgae *in vitro* condition in petri plates and also in batch. Therefore the samples were first cultured in source water then these were subcultured using Bolds Basal medium by making media in 60% source water and 40% distilled water. Later subculture was done by replacing the media with 40%, 30% 10% and 0% source water with BB media pH 6.8 at room temperature. The Bolds basal medium stock was prepared and utilized for fresh water algae culture. Stock solution was stored in amber coloured bottle at 4 $^{\circ}$ C in refrigerator.

3.3.1 Algae inoculated in solid bold basal medium:

- Solid BB medium was prepared by adding 10ml stock solutions of KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaNO_3 , K_2HPO_4 , NaCl and 1ml stock solutions of H_3BO_3 , trace metal solution, combination of EDTA+KOH and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \text{H}_2\text{SO}_4$, respectively.
- The pH 6.8 was adjusted and confirmed by digital pH meter, made the volume up to 1 litre by adding distilled water.
- Media was solidified by adding 0.8% of agar and keeping in autoclave at 121 $^{\circ}$ C, 15psi for 15 min.

- The media was poured to the autoclaved petri plates and kept aside to solidify. In the petri dishes on the solidified media all the seven samples were inoculated in the laminar airflow and the petri plates were air tightened with paraffin film.
- The inoculated petri dish was incubated in incubation chamber at temperature $27^{\circ}\pm 2^{\circ}$ and 16hr light. Three weeks later subcultured to new freshly prepared media and were used for microscopy study.



Figure 3.4: Multiplication methods of terrestrial microalgae,
(A) Petri plate method, (B) Algae Batch culture inside incubating orbital shaker,
(C) Growth of microalgae during batch culture after 15th and 20th day post culture,
(D) Incubating orbital shaker.

3.3.2 Batch culture:

In batch culture algal sample were grown in 250ml of Erlenmeyer flask with BB medium. The flask was kept in the orbital shaker at 120rpm at the room temperature 30°C . Additional medium was supplemented once in a two week during batch culture. The optical density was measured at the interval of 5 days and algae biomass was collected from medium by centrifuging at 10000rpm for 5min.

3.3.3 Growth kinetics of terrestrial microalgae:

Growth kinetics of the terrestrial microalgae samples were determined by the method described by Stanbury *et al.* (1997).

The growth kinetics is used to study the growth of a microorganism by measuring the turbidity by optical density using spectrophotometer and dry weight of algae cultured sample. Growth kinetics was studied by culturing the 7 different location algae sample in BB medium without agar. The optical density of that culture was read every 5 days interval at 600nm and dry weight was also taken once in every 5 days. Dry weight was taken by centrifuging 10 ml of media, drying the pellets in hot air oven at 60°C and weighing the algae biomass in centrifuge tube. Microalgae culture was kept in orbital shaker at the rate of 120rpm as to allow algae cells distribute equally in the culture medium which prevents the error of growth kinetics. Good biomass yield was recovered with this method.

3.3.4 Multiplication of algae in plastic tray:

Collected algae were kept for multiplication under direct sunlight in plastic tray using 20% source water and 80% normal tap water and kept it under direct sunlight. Evaporation was controlled by fixing 50% shade net and maintaining the water level by adding extra normal water for every 2 days interval.

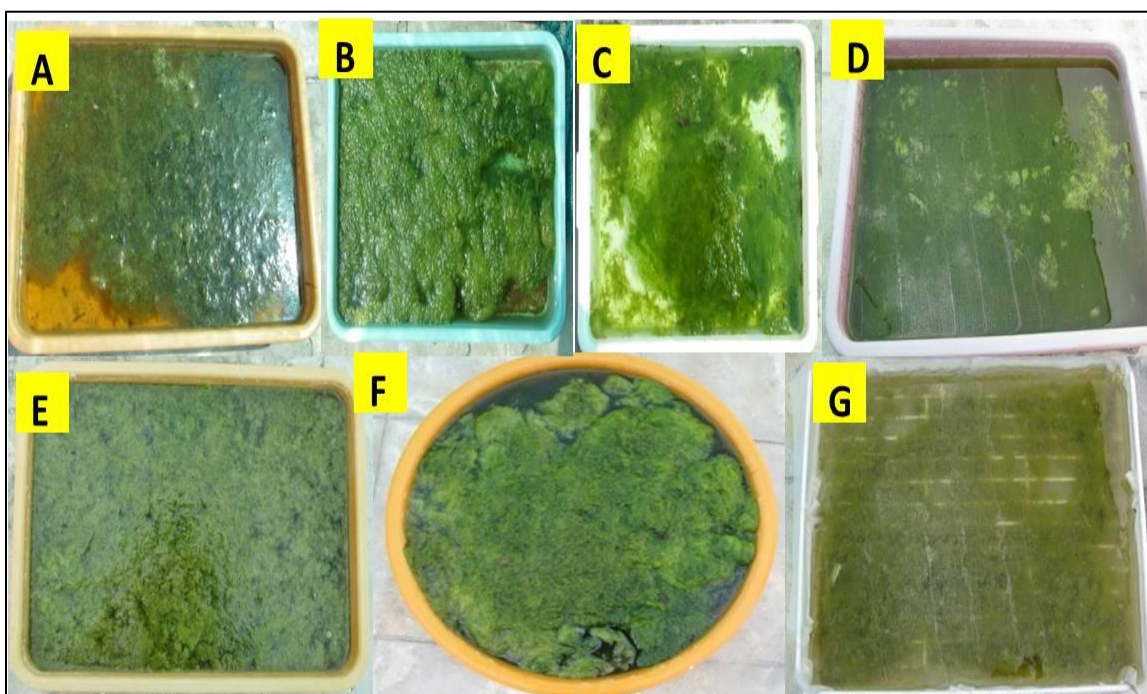


Figure 3.5: Multiplication of terrestrial microalgae in plastic tray.

**(A) Happanadka, (B) Dandiganahalli, (C) Hoovnalli, (D) Ikola, (E) ACH Campus,
(F) Kaggodu and (G) Gadanahalli**

3.3.5 Closed photobioreactor systems: *in vitro* method for multiplication of terrestrial microalgae:

Photobioreactor was made using 2 liters plastic transparent bottles. Photobioreactor was filled three fourth volumes with bold basal medium for the growing of microalgae. Sparger was developed for continuous agitation and exchange of air using DC motors. Artificial light was provided using white fluorescent lamp intensity $\approx 2500\text{Lux}$ for photosynthesis of algae at room temperature. For seven days interval 10ml of bold basal medium added to each setup. Growth of the microalgae in this method was measured by observing the optical density at 600nm using spectrophotometer.



Figure 3.6: Mass multiplication of Terrestrial microalgae by closed photobioreactor

- (A) Photobioreactor setup with BBM, (B) Photobioreactor setup with CO₂ gas supply
(C) Photobioreactor setup with sparger, (D) Photobioreactor setup with light.**

3.3.6 Open pond production method for multiplication of terrestrial microalgae:

Small ponds were opened in the dimension of 4×5×2 feet and covered with low density plastic sheets to prevent the leaching of stored water for the culture of microalgae. Combination of different types of water viz, normal tap water, water with nutrient supplements, R.O. residual dispense water whose pH 8.4 and urban waste water was used. To these different treatments of water seven identified microalgae samples were grown. To reduce the errors in open field 3 replication for every treatment was done. Provided 50% shade with green shade net to prevent the evaporation of water from ponds. In the small ponds for each treatment 10grams of wetted terrestrial collected microalgae was added and once in two days agitation was done manually for equal distribution of microalgae in the pond. Calculated amount of BBM were added to the nutrient supplements treated water as a nutrient supplements.



Figure 3.7: Layout of pond for different treatment for mass multiplication of terrestrial microalgae at College of Agriculture Hassan.

Where a = Normal tap water, b = water with nutrient supplements (BB medium), c = waste water from the water purifier, d = urban waste water and 1,2,3,4,5,6,7 were algae samples from Ikola, Gadanahalli, Happanadka, Hoovnalli, Kaggodu, Dandiganahalli and ACH campus respectively.

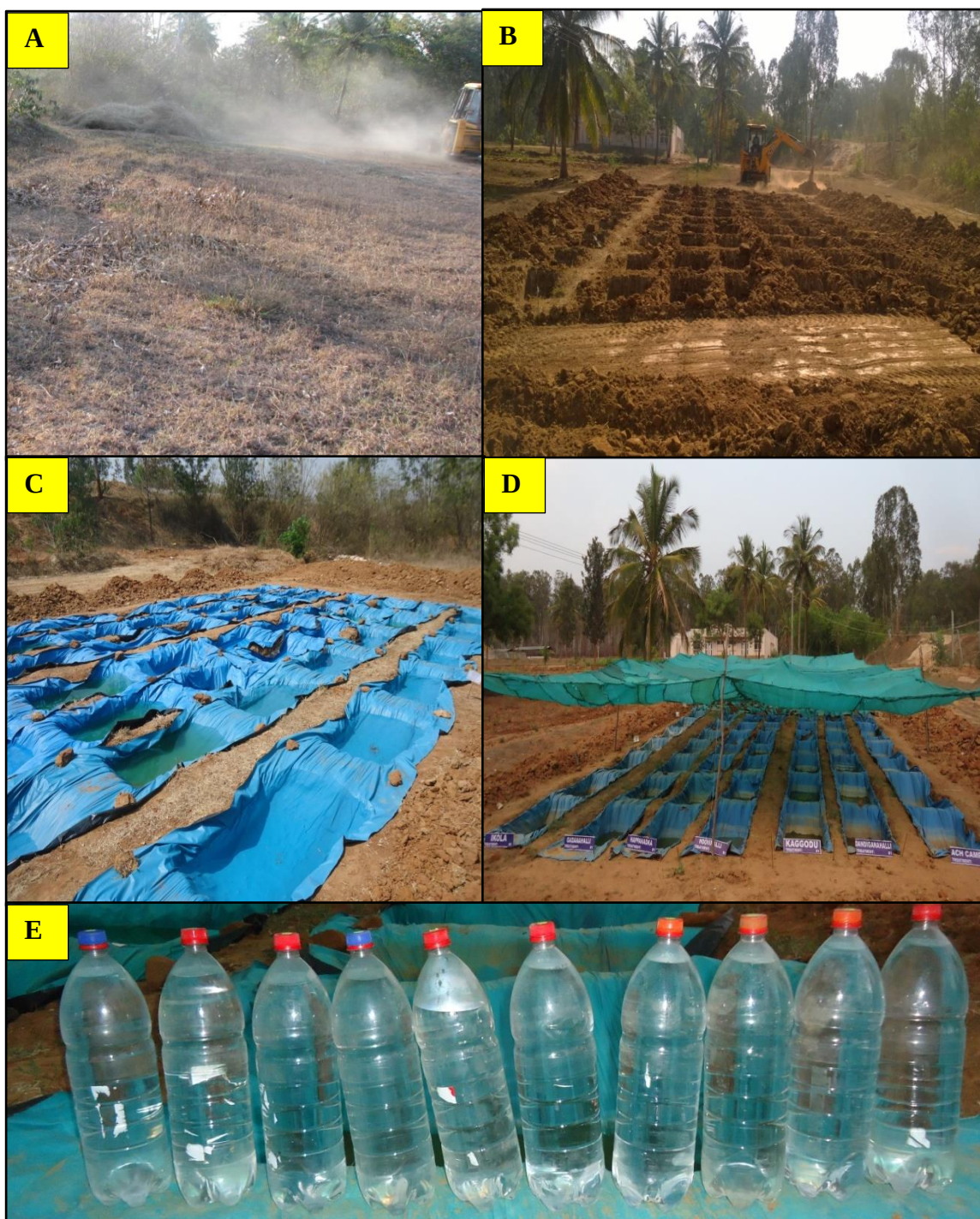


Figure 3.8: Mass multiplication of terrestrial microalgae in open pond system.

Where (A) Leveling of field, (B) Opening of pond by digging, (C) Low density plastic sheets filled with water, (D) Open pond shaded with green shade net, (E) BBM stocks for water with nutrient supplements.

3.3.7 Harvesting of microalgae:

Microalgae were harvested by two methods in this experiment such as centrifugation and biomass filtration method. According to **Mohn (1980)** biomass filtration was economical, compared to centrifugation method.

3.3.7.1 Centrifugation method:

Harvesting of algae was done by the method described by **Brennan and Owende (2010)**.

It is an efficient method for separation of solid/liquid with the different rpm. For centrifugation, collected algae samples were filled in centrifuge tubes and centrifuged at a rate of 10000rpm for 10min. The supernatant was discarded and the pellet was collected for further study.

3.3.7.2 Biomass filtration:

The method of **Mohn (1980)** was followed for biomass.

A conventional filtration process is most appropriate for harvesting of relatively large size microalgae such as *Spirulina*. In this method collected algae were filtered by conventional filtration method using sieves whose size 0.2mm. This method easily removes the excess water from the microalgae. It was an easy method for harvesting the large quantity microalgae.

3.3.8 Drying of microalgae under sun light and shade:

- Biomass filtered algae was dried by exposing to direct sunlight and observed, it took less time.
- In shade drying it took more time to dry than under sun.
The drying was performed on blotting towels under shade in laboratories.

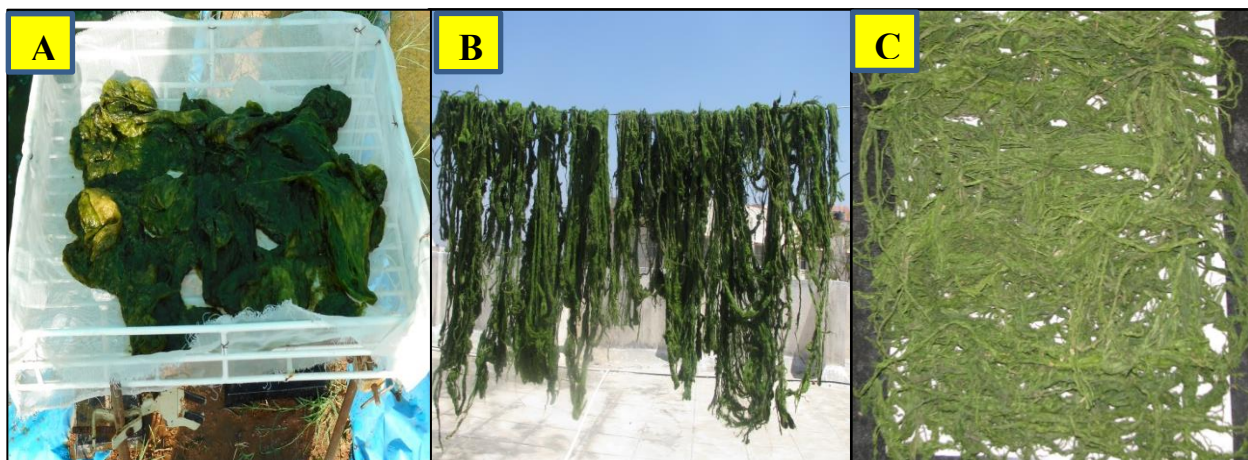


Figure 3.9: Harvesting and drying of terrestrial microalgae.
(A) Biomass filtration, (B) Sun drying and (C) Shade drying

3.4 Biochemical characterization of selected species of terrestrial microalgae:

Biochemical characterization of terrestrial microalgae was done by estimating chlorophyll, carbohydrates (total soluble sugars and total reducing sugars), protein and lipid contents.

3.4.1 Chlorophylls estimation from terrestrial microalgae:

Chlorophyll (a, b, c and total) estimation was done by method described by **Parsons (1963)** and **Sadasivam and Manickam (1992)**.

Reagent:

- Analytical grade acetone (80%).

Procedure:

- Weighed 1g of algal samples of different location separately.
- Ground the tissue using liquid Nitrogen in pestle and mortar
- Made a fine pulp with the addition of 20ml of 80% acetone.
- Centrifuged at 6000rpm for 10min and transferred the supernatant to a 100ml volumetric flask.
- Repeated this process until the residue of the sample colourless with addition of 80% acetone, centrifuged and transferred the supernatant to the volumetric flask.
- Washed the pestle and mortar with 80% acetone and collected the washings in the same volumetric flask.
- Made up the volume of supernatant to 100ml with 80% acetone.
- Read the absorbance of the solution at 645, 663 and 450nm against the solvent and 80% acetone used as blank.
- For chlorophyll c first read the sample at 450nm and noted the absorption value and
- Next added one drop of concentrated HCl to same sample and read the absorbance at 450nm.

Chlorophyll content in terrestrial microalgae was calculated by using the formulae given below:

- **Chlorophyll a/g tissue (mg) = $12.7(A_{663}) - 2.69(A_{645}) \times \frac{V}{1000 \times W}$**
- **Chlorophyll b/g tissue (mg) = $22.9(A_{645}) - 4.68(A_{663}) \times \frac{V}{1000 \times W}$**
- **Total chlorophyll /g tissue (mg) = $20.2(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$**
- **Chlorophyll c/g tissue (mg) = $(A_{450} - A_{450 \text{ with HCl}}) \times 17.5$**

Where A = absorption at specific wavelengths, V = Final volume of chlorophyll extracted in 80% acetone (100ml) and W = fresh weight of tissue extracted.

3.4.2 Estimation of total soluble sugar for biochemical characterization and fermentation study:

Estimation of total soluble sugar was done by following the method described by **Sadsivum and Manickam (1992)** and **Lalitesh *et al.* (2017)**.

Reagents:

- HCl (2.5N): It was prepared by slowly adding 20.529ml of HCl to 25ml distilled water and adjust the final volume of solution to 100ml.
- Anthrone reagent: dissolved 200mg Anthrone in 100ml of ice cooled 95% H₂SO₄. Prepared freshly just before use.
- Standard glucose stock: dissolved 100mg of glucose in 100ml of water.
- Working standard - 10ml of stock diluted to 100ml with distilled water (100µg/ml).

Procedure:

- Weighed 100mg of algal sample and placed it into a boiling tube.
- Hydrolysed by keeping it in a boiling water bath for 3hrs with 5ml of 2.5N HCl and cooled to room temperature.
- Neutralized by adding solid sodium carbonate until the effervescence cases.
- Made up the volume to 100ml by adding distilled water
- Centrifuged above solution with 10000 rpm for 10min.
- Collected the supernatant and taken 0.2 and 0.4ml aliquot for analysis.
- Prepared the standards by taking 0.2, 0.4, 0.6, 0.8 & 1ml of working standard. “0”ml served as blank.
- Made up the volume to 1ml in all tubes including the sample tubes by adding distilled water.
- Then added 4ml Anthrone reagent.
- Kept it in boiling water bath for 8min.
- Cooled rapidly and measured the green to dark green colour at 630nm using spectrophotometer.
- Drew the standard graph by plotting concentration and standards on the x-axis versus absorbance of y-axis. From the graph calculated the amount of carbohydrates present in the sample tube extending.

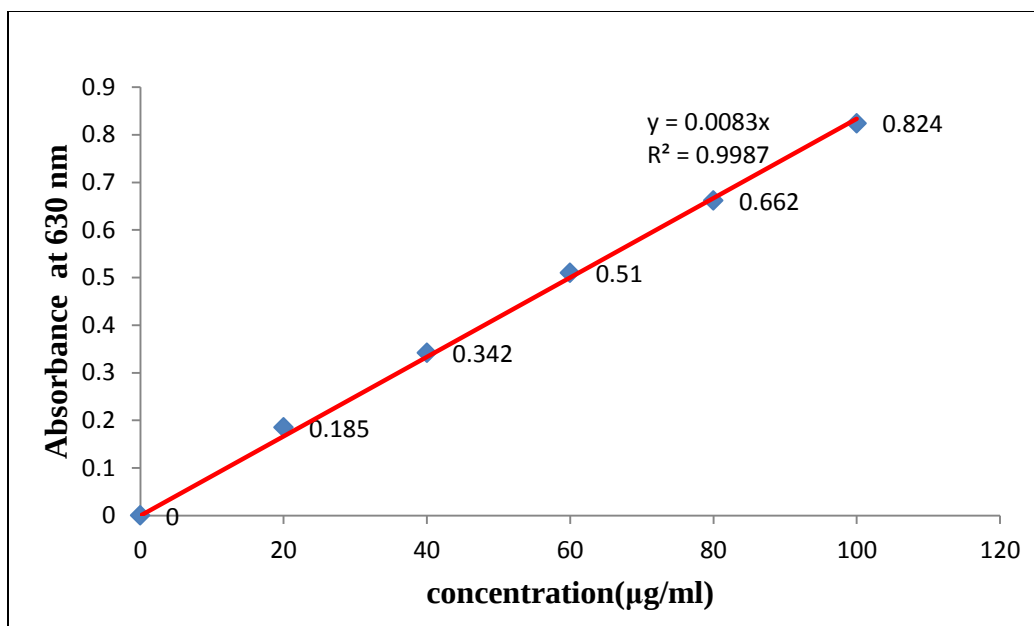


Figure 3.10: Standard graph of total soluble sugar prepared using glucose

Total soluble sugars content in terrestrial microalgae was calculated by using the formulae given below:

$$\text{Amount of Carbohydrates present in sample (\%mg)} = \frac{\text{Sugar value from graph (mg)}}{\text{Aliquot sample used (0.2ml)}} \times \frac{\text{Total volume of extract (ml)}}{\text{weight of sample (mg)}} \times 100$$

3.4.3 Estimation of reducing sugar for biochemical characterization and fermentation study:

Estimation of reducing sugar was done by following the method described by **Miller (1972)** and **Lalitesh *et al.* (2017)**.



Reagents:

- Dinitrosalicylic acid (DNS) reagent: Dissolved simultaneously 1g of DNS acid, 200mg of crystalline phenol and 50mg of sodium sulphite in 100ml of 1% NAOH solution by stirring.
- 40% Rochelle salt solution (sodium-potassium tartarate solution): 40g of sodium-potassium tartarate dissolved in 100ml of distilled water.
- Standard glucose solution :50mg of glucose dissolved in 50ml distilled water (1mg/ml)

Procedure:

- Weighed 100mg of the sample and extracted the sugars with hot 80% alcohol twice (5ml each time). Centrifuged above solution with 10000 rpm for 10min.
- Collected the supernatant and evaporated on water bath.
- Added 10ml of water to dissolve the sugars.
- Pipetted out aliquots of 0.2ml to a test tube and made up the volume to 1ml with distilled water
- Added 3ml of DNS solution then heated the test tube with sample until develop a colour (10min) in a boiling water bath
- After the colour has developed added 1ml of 40% Rochelle salt solution and mixed it in vertex mixture.
- Cooled the tubes and measured the absorbance at 510nm using spectrophotometer.
- Calculated the amount of reducing sugar in the sample using standard graph.
- It is a convenient and sensitive method for the estimation of reducing sugars, particularly when large numbers of samples are to be analyzed.
- This method is not suitable for determination of a complex mixture of reducing sugars as the standard curves do not always pass through the origin and different sugars give different color yields

Total reducing sugar content in terrestrial microalgae was calculated by using the formulae given below:

$$\text{TRS (\%mg)} = \frac{\text{Sugar value from the graph (\mu g)}}{\text{Equation of straight line on standard graph} \times \text{Aliquot of alcohol free extract concentration (mg)}} \times 100$$

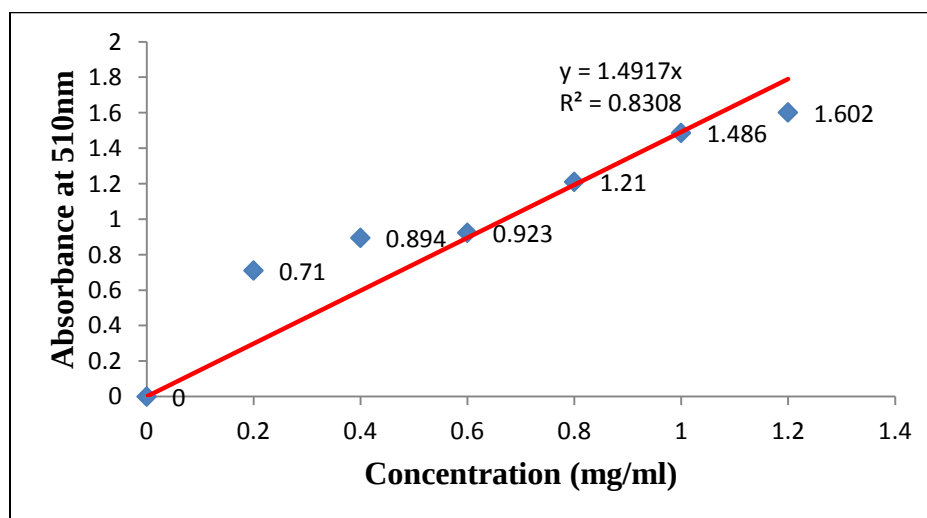


Figure 3.11: Standard graph of total reducing sugar prepared using glucose

3.4.4 Estimation of protein for biochemical characterization and fermentation study:

Estimation of protein was done by following the method described by **Lowry *et al.*, (1951)**.

Reagents:

- Solution A: Sodium carbonate (2%) in NaOH (0.1N). Dissolved 2g Na_2CO_3 in 100ml of 0.1N NaOH. NaOH was standardized with HCl solution.
- Solution B: Prepared just prior to use by mixing equal volume of 1% copper sulphate solution and 2% sodium potassium tartarate (Rochelle salt) solution.
- Solution C: Alkaline cupric tartarate solution. Prepared just prior to its use by mixing 50ml of solution A and 1ml of solution B.
- Solution D: Folin-Ciocalteu reagent (1N). Generally FCR (Phosphomolybdate and phosphotungstate) available commercially is in 2N. Diluted commercial reagent (2N) by adding equal volume of water to form 1N reagent. Prepared just prior to its use.
- Stock standard protein solution: 50mg of bovine serum albumin/50ml of water.
- Working standard solution: Diluted 10ml of the stock solution to 50ml with water to obtain 200 μg protein/ml.
- phosphate buffer (0.1M, pH 7.4)
- β - Mercapto ethanol (2 μl).
- Polyvinylpyrrolidone (2%)

Extraction of protein from sample:

- Ground 0.5gm of the Algal sample with 5ml of phosphate buffer (0.1M, pH 7.4) in a pestle and mortar and added pinch of polyvinylpyrrolidone (and 2 μl β mercapto ethanol and centrifuged at 6000rpm for 10min.
- Collected the supernatant and added equal amount of chilled acetone and kept at -20°C for 30min.
- Then once again centrifuged at 12000 rpm for 15min.
- Discarded the supernatant and washed the pellet with 75% ethanol until white pellet appears.
- Then dissolved the pellet in 1ml of phosphate buffer (0.1M, pH 7.4) and used it for protein estimation.

Estimation of protein from sample:

- Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0ml of working standard solution into series of test tubes.
- Pipetted out 0.2ml and 0.4ml of the sample extract into two other test tubes.
- Made up the volume to 1ml with water in all test tubes. A tube with 1ml of water served as blank.
- Added 5ml of solution C mixed well and incubated at room temp for 1min.
- Added 0.5ml of FCR mixed well immediately and incubated at room temp in dark for 30min.
- Observed the absorbance at 660nm against the blank using spectrophotometer.
- Drew the standard graph, calculated the amount of protein in the sample and express the results as mg/g or mg/100g sample or percentage.

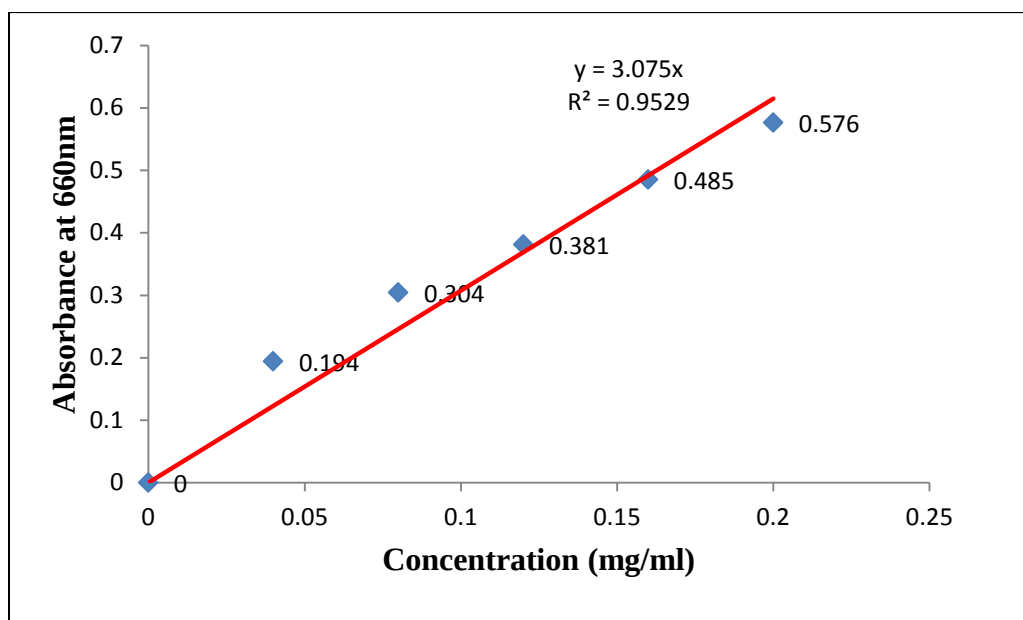


Figure 3.12: Standard graph of protein prepared using BSA.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE) for protein:

SDS - PAGE was done by following the method described by **Sambrook, J. and Russell, D. W. (2001)**.

- Acrylamide and bis acrylamide at 29:1 was prepared as stock.
- For gel run resolving gel and stacking gel was prepared with different gel concentration and pH.
- SDS (10%) was prepared in autoclaved double distilled water.
- APS (10%) prepared freshly before use by dissolving 100mg APS in 1ml sterile water.
- Resolving gel (10%) and stacking gel (5%) were also prepared.

Table 3.2: SDS - PAGE recipes for protein

Particles	Gel Resolving (30ml)	Staking Gel (10ml)
Water	11.9ml	3.4ml
30% acrylamide mix	10ml	0.83ml
Tris buffer (1.5M, pH8.8)	7.5ml	-
Tris buffer pH(1.0M, 6.8)	-	0.63ml
10%SDS	0.3ml	0.05ml
10%APS	0.3ml	0.05ml
TEMED	0.012ml	0.005ml

Casting and loading gel

- 15 ml resolving gel was prepared and loaded.
- 1 ml of isopropanol was added in casting caset of Life technologies mini gel tank to remove the bubbles and the gel surface uniformly spreads.
- After solidification of gel, removed the isopropanol by washing with double distilled water.
- Then added 5 ml of staking gel and set the comb for well formation
- After solidification of gel the comb was removed.
- Loaded the protein sample in well.

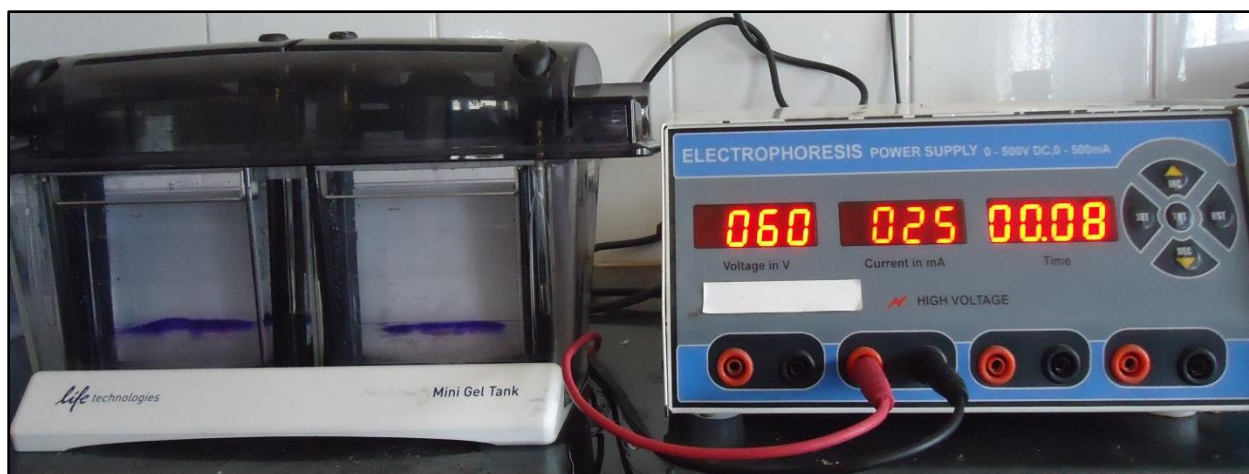


Figure 3.13: Compact mini gel setup with power pack used for PAGE.

Care needed to take during SDS – PAGE running for protein:

The following precautions should be taken during electrophoresis

- SDS dye should be added to protein sample to increase the density otherwise it will not settle.
- For 50µl added 10µl of dye (5X).
- Gel set up kept in the buffer tank containing SDS buffer then connect the power pack
- After 2 hours of gel run staining was done with staining solution and after 12 hours destaining was done with destaining solution until bands in the gel looks clearly.
- Then visualized the protein bands.

3.4.5 Lipid extraction from terrestrial microalgae:

Algae lipid extraction was done by following the method of **Pratoomyot *et al.* (2005); Shalaby (2011) and Sadsivum and Manickam (1992)**

Reagents:

- Petroleum ether/Hexane and methanol (2:1)

Procedure:

- Weighed 10 gram of hot air oven dried at 55-60°C algal sample in a thimble (prepared from Whatman no. 41 filter paper) and placed it in the Soxhlet apparatus.

- Connected a dry pre-weighed solvent mixture flask ('a'g) beneath the apparatus and added the required volume of solvent and connected to the condenser.
- Adjusted the heating rate of the apparatus using heat regulators to 65°C for 4 hours and allowed to siphoning till 16-18 times.
- Removed the thimble and retained petroleum ether mixture from the apparatus by distillation method.
- Evaporated the excess of petroleum ether from the solvent flask on a hot water bath and dry the flask at 90°C for 15min.
- Cooled the flask in a desiccator and weighed ('b'g).

Crude oil content in terrestrial microalgae was calculated by using the formulae given below:

$$\text{Crude oil content in sample (\%)} = \frac{(\text{b}-\text{a})}{\text{weight of the sample (g)}}$$

Where 'a' was a pre weighed dried round bottom flask

'b' was a flask with oil/lipid

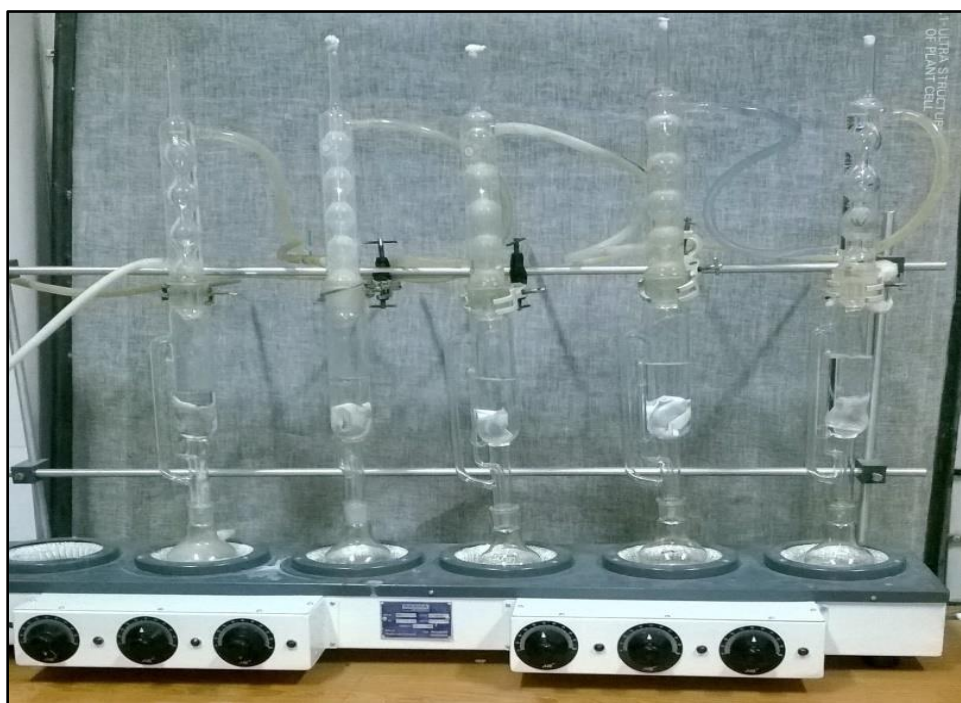


Figure 3.14: Soxhlet apparatus used for crude oil sample extraction from micro algae

3.5 Transesterification of microalgal oil for biodiesel production:

Transesterification of microalgae was done by following the method described by **Shalaby (2011); Stergiou *et al.* (2013)** and **Saber *et al.* (2016)**.

Transesterification is a multiple step reaction, including three reversible steps in series, where triglycerides are converted to diglyceride, then diglycerides are converted to monoglycerides, and monoglycerides are then converted to fatty acid esters (biodiesel) and glycerol (byproduct).

Preparing the Methoxide solution:

- Measured 110 ml methanol and placed into 1L jar. Closed the jar with the lid and tighten securely.
- Measured 2 grams NaOH and quickly added it into the methanol, minimizing its exposure to air. Again closed the jar with the lid and tighten securely. Label the jar “Danger: Methoxide” along with the amount of methanol and NaOH.
- Enough time, was given to the catalyst to dissolve in the methanol, though gentle agitation helps. Made sure that all of the solid catalyst had dissolved in the methanol before proceeding. Care was taken as, the methoxide solution just created is the most dangerous substance used in biodiesel production.

Transesterification of terrestrial microalgae oil for biodiesel production:

The standard ratio of lipid to methoxide is 500ml of lipid in 110 ml of methoxide; same ratio was used in the study

- 2ml of microalgal oil was taken and added 0.44μl of methoxide in 3ml tubes.
- Using dry bath with shaker heated the oil to 60°C and shakes the tube vigorously at the rate of 200rpm for 1hr.
- It formed two separate layer in which upper layer was biodiesel and lower layer was glycerol (byproduct).
- The tubes were kept in warm place and left to allow settling.
- After that separate the upper layer which contain biodiesel and washed with warm water 4 to 5 times to remove the impurities and excess glycerol.

3.6 Bioethanol production by fermentation of Terrestrial microalgae:

The method of **Harun *et al.* (2014)** was followed.

Bioethanol production study was attempted by anaerobic fermentation using defatted algal residues. The defatted algal residue was acid hydrolyzed with HCl (2.5N) and was neutralized with Na₂CO₃. This hydrolyzed residue was used as substrate for fermentation using 5% of *Saccharomyces cerevisiae* inoculum. In fermentation setup every three days interval biochemical components viz, percent alcohol, total soluble sugars, total reducing sugars, proteins, pH and microbial load were estimated and used for standardization of fermentation time.

3.6.1 Fungal culture for fermentation study:

- The yeast (*Saccharomyces cerevisiae*) is known for the production of Ethanol. Preserved cultured *Saccharomyces cerevisiae* was collected from the laboratory of biochemistry department of Agricultural biotechnology block, Agriculture College Hassan sub campus of UAS, Bangalore.

3.6.2 Preparation of algal hydrolyses:

- Defatted algae were ground to fine powder in pestle and mortars and crushed algae were autoclaved at 121°C for 15min at 15Psi.
- Cooled the sample and that autoclaved crushed algae were homogenized treated with HCl (2.5N) for 3hrs, which helps to conversion of polysaccharides into monosaccharides.

3.6.3 The experimental set up was done for fermentation:

- In this experiment defatted algal residue hydrolyzed with 2.5N HCl was used as substrate for fermentation process.
- Yeast (*Saccharomyces cerevisiae*) was used for breaking the sugar contents in the algal residue to produce ethanol.
- Sterilized double distilled Water, conical flask, rubber cork with pipes were used for anaerobic fermentation setup.
- Here 7 defatted 10g of hydrolyzed algae from various places were inoculated with 5% *S. Cerevisiae* and 0.5% sucrose were used as a starter for this experiment. 90ml of sterilized water was added and sample was taken for initial analysis that was considered as 0th day sample. The flasks were closed with rubber cork.
- Conical flasks were airtight by rubber cork fixed with 2 silicon pipes among two one medium sized silicon pipes was immersed to a test tube with water to ensure anaerobic condition. Another small pipe connected to stopper and syringe for sampling the sample in the interval of 3 days for experimental 8 parameter analyses with. The stoppers fixed checks the air entry to the anaerobic condition. This experiment was setup in 3 replications R₁, R₂ and R₃ to minimize errors.

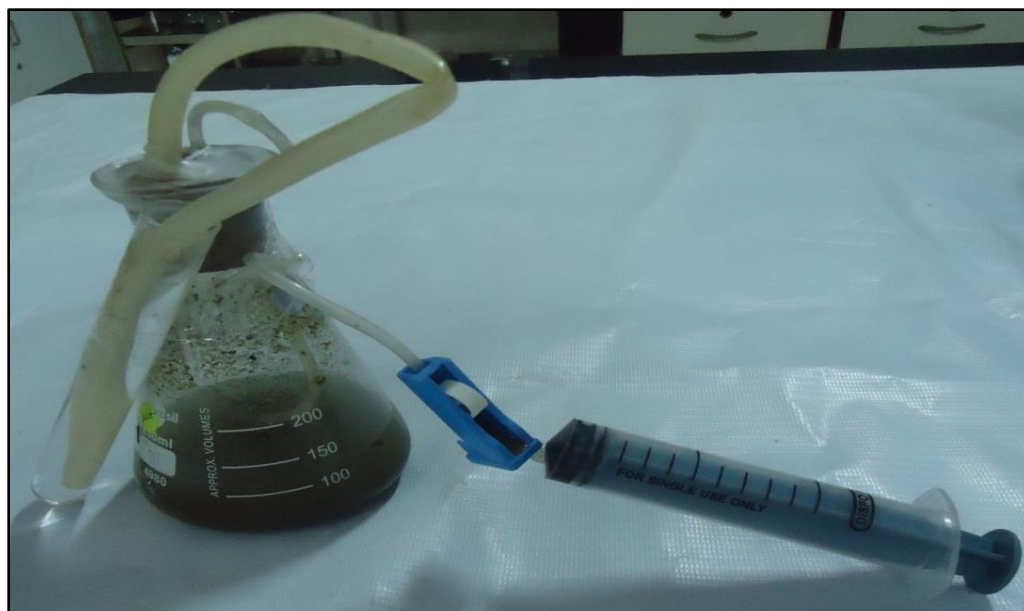


Figure 3.15: Anaerobic fermentation study setup used for acid hydrolyzed microalgae



Figure 3.16: Anaerobic fermentation setup with different treatment from place of collection.

3.6.4 Determination of pH of the fermentation setup of terrestrial microalgae:

- **Apparatus:** pH meter, standard flasks, magnetic stirrer, funnel, beaker, wash bottles, tissue paper, forceps.
- **Reagents:**
 - Buffer solutions (pH 4.01, 7.0 & 9.2)
 - Potassium chloride

Testing of sample:

- In a clean dry 100ml beaker taken the sample and placed it in a magnetic stirrer, inserted the Teflon coated stirring bar and stirred well.
- After that placed the electrode in the beaker containing the sample and checked for the reading in the pH meter. Wait until you get a stable reading and noted the reading showed in pH meter.
- Taken out the electrode from the sample, washed it with distilled water and then wiped gently with soft tissue paper.

3.6.5 Estimation of Microbial load of the fermentation setup of terrestrial microalgae:

Measurement of Cell Concentration in Suspension by Optical Density:

- Light is passed through the suspension of microorganisms, and all light that is not only absorbed but also is re-radiated, involves Huygens' Principle (a good choice is Light Scattering by Small Particles).
- Light passing through a suspension of microorganisms is scattered, and the amount of scatter is an indication of the biomass present in the suspension.
- Measurement of Cell Concentration in Suspension by Optical Density at 600nm.

Procedure:

- Taken 1ml of sample from the anaerobic setup.
- Made up the volume to 2ml by adding distilled water.
- Shaken well and leave it for 5 min.
- Measured the absorbance at 600nm using spectrophotometer.

3.6.6 Alcohol estimation:

After fermentation, the fermentation broth was filtered and distilled at 79°C because boiling point of ethanol is 78.37°C. Estimation of alcohol was done by following the method described by **Williams *et al.* (1950)** and **Stanbury *et al.* (1997)**.

Reagents preparation:

- **Potassium dichromate:** 34g of potassium dichromate was dissolved in 500ml of distilled water and 325ml of sulphuric acid then volume made up to 1000ml with distilled water that give 0.23N $K_2Cr_2O_7$.
- **Preparation of stock solution:** Standard stock solution of 100% pure analytical grades (containing 789mg/ml) ethanol was prepared by dissolving 12ml of ethanol in 100ml distilled water which resulted in 100mg/ml of standard ethanol.

Materials required:

Test tubes, pipette, conical flask, round bottom flask, condensers, distillation unit.

Procedure:

- 1ml of anaerobic maintained algae sample from each treatment was transferred to 250ml round bottom distillation flask connected to the condenser and diluted with 30ml of distilled water. The sample was distilled at 74-75°C.
- The distillate was collected in 25ml of $K_2Cr_2O_7$ (0.23N) reagents kept at receiving end.
- The containing alcohol was collected till total volume of 45ml was obtained.
- Similarly standard (20-100mg ethanol) that is standard containing a 0.2, 0.4, 0.6, 0.8, & 1ml of stock solution were mixed with 25ml of $K_2Cr_2O_7$ separately. Distillate the sample and standards were heated in water bath at 60°C for 20 minutes and cooled.
- The volume made up to 50ml with distilled water and absorbance was measured at 600nm using spectrophotometer.
- The standard curve was plotted considering the concentration against absorbance.

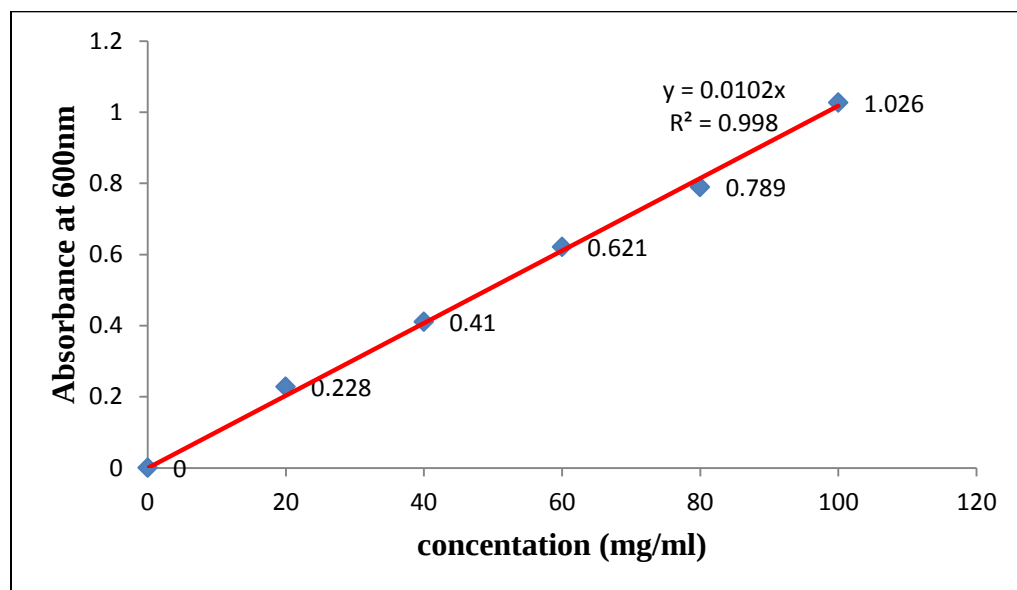


Figure 3.17: Standard graph of ethanol prepared using ethanol.

3.7 Effects of terrestrial microalgae on treated water:

The following method of **H.S. Industries Multi parameter water testing field kit, and fallowed their procedure (Technology transfer from BARC, Mumbai)** was adopted for estimation of Chloride, Iron and Nitrate in the treated water samples with algae.

3.7.1 Chloride estimation:

- Pipetted 5ml of water sample in a test tube
- Added 5 drops of chloride reagent A and mixed with Cyclo mixer
- Then added chloride reagent B drop by drop and counted the number of drops till brick red colour appears.
- Calculated the chloride content in the water based on formula given below

Chloride = Number of drops of reagent B added $\times$ 10ppm as Cl

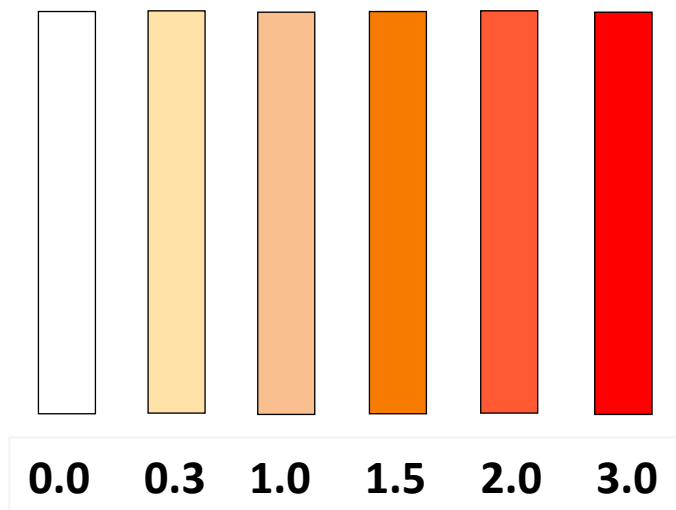


Figure 3.18: Colure bar chart representing iron coinciding values (ppm)

3.7.2. TDS estimation:

- Pipetted 10ml of water sample in a beaker.
- Dipped the TDS meter in the beaker containing water sample
- Noted the TDS reading of the sample

3.7.3. pH estimation:

- Pipetted 10ml of water sample in a beaker.
- Dipped the pH meter in the beaker containing water sample
- Noted the pH reading of the sample



Figure 3.18: Multiple parameter water testing field kit from H.S. Industries

3.9 Statistical analysis:

The data recorded during the course of investigation were subjected to statistical analysis by “Analysis of variance” technique (**Fisher and Yates, 1963**) for drawing conclusions. The significant and non-significant treatment affect was tested against the critical difference at 5% level. The significant difference between the means was tested against the critical difference at 5% level. For testing the hypothesis, the following ANOVA table was used. The skeleton of analysis of variance ANOVA is furnished in the given **Table 3.3**.

Table 3.3: Skeleton of ANOVA table to be used in the statistical analysis

Source of variation	d.f.	S.S	M.S.S	F(Cal)
Due to treatment	(t-1)	TSS	TSS/(t-1)	TMS/EMS
Due to replication	(r-1)	RSS	RSS/(r-1)	RMS/EMS
Due to error	(r-1)(t-1)	ESS	ESS/(r-1)(t-1)	
Total	(rt-1)	TSS		

$$S.E(d) = \sqrt{2} \times \bar{E}MSS/r$$

$$CD = SE(d) \times t_{\alpha/2, r(t-1)} \times \sqrt{\text{degree of freedom at 5\% levels of significance}}$$

RESULT AND DISCUSSION

Present study entitled “**Biodiesel and bioethanol production from the terrestrial micro algae and their by-products**” were investigated and data obtained are hereby discussed in the following sub headings:

4.1 Lipid content of terrestrial microalgae collected from different locations

In this study, around 62 terrestrial algae were collected from the fresh water bodies of in and around of the Western Ghats regions (**Table 4.1**). These collected algae were identified based on microscopic and biochemical characteristics of *Prescott (1970)* and *Sushanto et al. (2015)*. Maximum oil content found in the sample collected from the Dandiganahalli was 43 % and minimum found in the Kerveshe was 2.15 % of dry weight of sample. Out of these only 7 samples were identified as high oil content and biomass yielding microalgae and used further for multiplication and fermentation study.

Table 4.1: Lipid content of terrestrial microalgae collected from different locations of in and around Western Ghats of Karnataka state.

Sl.No	Place	Source	Oil %
01	Hoskeri (Kodagu)	River	6.60
02	Hoskeri (Kodagu)	Pond	4.40
03	Chettali (Kodagu)	Pond	2.50
04	Chettali (Kodagu)	Open Well	7.20
05	Maragodu (Kodagu)	River	8.56
06	Maragodu (Kodagu)	Pond	6.40
07	Galibidu (Kodagu)	Open Well	3.70
08	Ikola Holley (Kodagu)	River	31.75
09	Cheyandane (Kodagu)	River	8.20
10	Nariyandada (Kodagu)	River	6.60
11	Shanmangala(Kodagu)	River	4.18
12	Kaggodu (Kodagu)	River	41.25
13	Kanthur Murnad (Kodagu)	River	9.30
14	Mekeri (Kodagu)	River	2.50
15	Kiggal (Kodagu)	River	6.80
16	Kattemadu (Kodagu)	River	3.60
17	Mudabidri (Dakshina Kannada)	River	11.80
18	Mudabidri (Dakshina Kannada)	Pond	8.30
19	Mudabidri (Dakshina Kannada)	Open Well	6.27
20	Beltangadi (Dakshina Kannada)	River	3.50
21	Ujjire (Dakshina Kannada)	River	6.70
22	Darmastala (Dakshina Kannada)	River	4.50

Contd.

23	Sanoor(Udupi)	River	13.65
24	Happanadka (Udupi)	River	31.25
25	Mudar (Udupi)	River	3.60
26	Shirlal (Udupi)	River	5.07
27	Ajekar (Udupi)	River	4.80
28	Kervashe (Udupi)	River	2.15
29	Arbi (Udupi)	River	9.31
30	Andaru (Udupi)	River	6.40
31	Karkala (Udupi)	River	4.70
32	Kundapura (Udupi)	River	3.30
33	Gokarna (Uttar Kannada)	River	2.50
34	Shrungeri (Chikamagaluru)	River	7.30
35	Koppa (Chikamagaluru)	Pond	5.80
36	Mudigere (Chikamagaluru)	Open Well	3.10
37	Mudigere (Chikamagaluru)	Pond	4.30
38	Ajjampur (Chikamagaluru)	Pond	10.65
39	Kaduru (Chikamagaluru)	Pond	8.32
40	Biruru(Chikamagaluru)	Pond	7.60
41	Madagada Kere (Chikamagaluru)	Pond	5.84
42	Dandigana Halli (Hassan)	Pond	43.0
43	Karekere (Hassan)	Pond	2.60
44	Heragu (Hassan)	Pond	5.80
45	K. Byadaralli (Hassan)	Pond	3.10
46	Udayapura (Hassan)	Pond	7.65
47	Shantigram (Hassan)	Lake	2.60
48	Aluru (Hassan)	Pond	7.50
49	Hoovnalli (Hassan)	Pond	32.75
50	Yaliyuru (Hassan)	Pond	6.50
51	Gadanahalli (Hassan)	Pond	36.0
52	Kavalikere (Hassan)	Pond	4.64
53	Kandli (Hassan)	Pond	3.80
54	Bhuvanalli (Hassan)	Pond	11.80
55	Malali (Hassan)	Pond	8.30
56	Kundoor Mata (Hassan)	Pond	3.75
57	ACH Campus (Hassan)	Tank	31.5
58	Aladahalli (Hassan)	Pond	4.22
59	Hittaladahalli (Hassan)	Pond	10.50
60	SHUATS Campus Allahabad (UP)	Pond	4.70
61	Rambagh Allahabad (UP)	River	7.30
62	Triveni Sangham Allahabad (UP)	River	7.90

The selected samples were from the location of Agricultural College Hassan (ACH) Campus (Hassan district), Dandiganahalli (Hassan district), Gadanahalli (Hassan district), Happanadka (Udupi district), Ikola (Kodagu district), Hoovnalli (Hassan district) and Kaggodu (Kodagu

district) were selected. In these seven samples, microalgae collected from Dandiganahalli was to have maximum (43%) lipid content and minimum (31.25%) in microalgae sample collected from Happanadka (**Figure 4.1**). Lipid content in the samples were varies mainly because of different species and biodiversity of terrestrial microalgae (**Metting, 1996**).

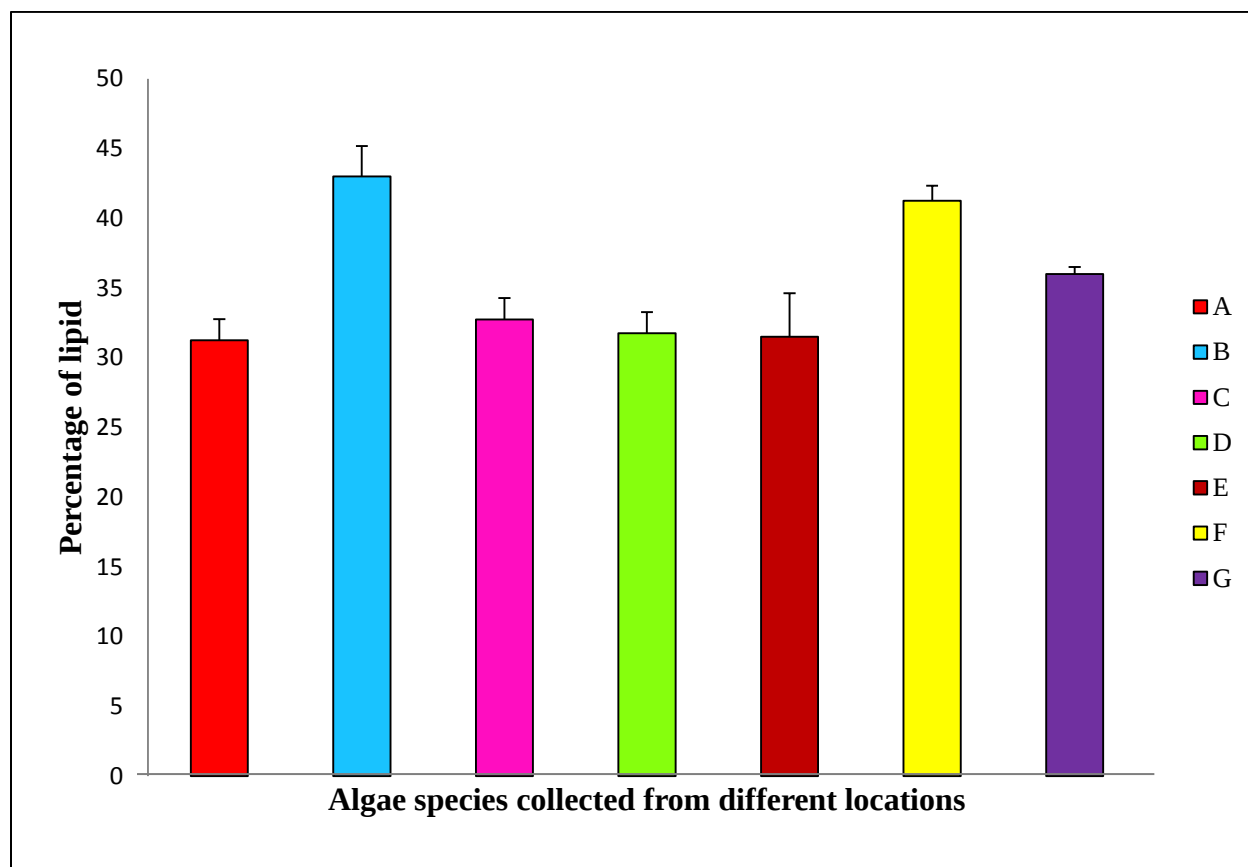


Figure 4.1: Total lipid content in the seven selected terrestrial microalgae collected from in and around the Western Ghats of Karnataka.

Where,

- A = *Rhizoclonium sp.* collected from Happanadka (Udupi district)
- B = *Rhizoclonium sp.* collected from Dandiganahalli (Hassan district)
- C = *Spirogyra* collected from Hoovnalli (Hassan district)
- D = *Klebsormidium sp.* Collected from Ikola (Kodagu district)
- E = *Cosmarium sp.* collected from ACH campus (Hassan district)
- F = *Rhizoclonium hieroglyphicum* collected from Kaggodu (Kodagu district)
- G = *Oedogonium sp.* collected from Gadanahalli (Hassan district)

4.2 Identification of Microalgae:

All the algae samples were identified by using compound microscope (40X and 100X) and scanning electronic microscope based on the reference of **Prescott (1970)**. The seven different species of terrestrial microalgae were rich in their oil content (**Table 4.1**), from fresh water were identified on the basis of morphological characteristics as *Cosmarium sp.*, *Klebsormidium flaccidum*, *Oedogonium sp.*, *Rhizoclonium sp.*, *Rhizoclonium hieroglyphicum* and *Spirulina sp.* These seven identified species were found to have variable lipid content due to biodiversity of species, habitat and location *etc.*

4.2.1 *Cosmarium* sp.

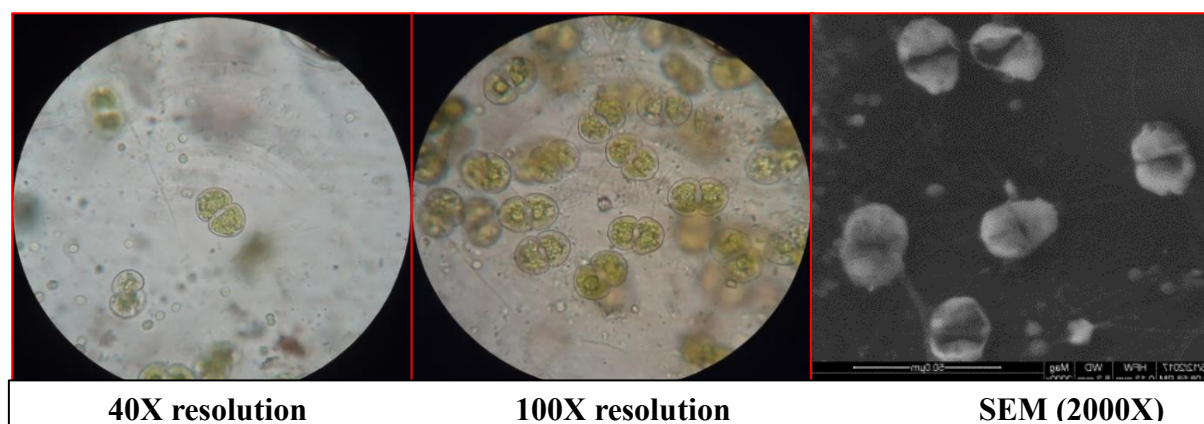


Figure 4.2: Microscopic identified *Cosmarium* sp. collected from ACH Campus.

4.2.2 *Oedogonium* sp.

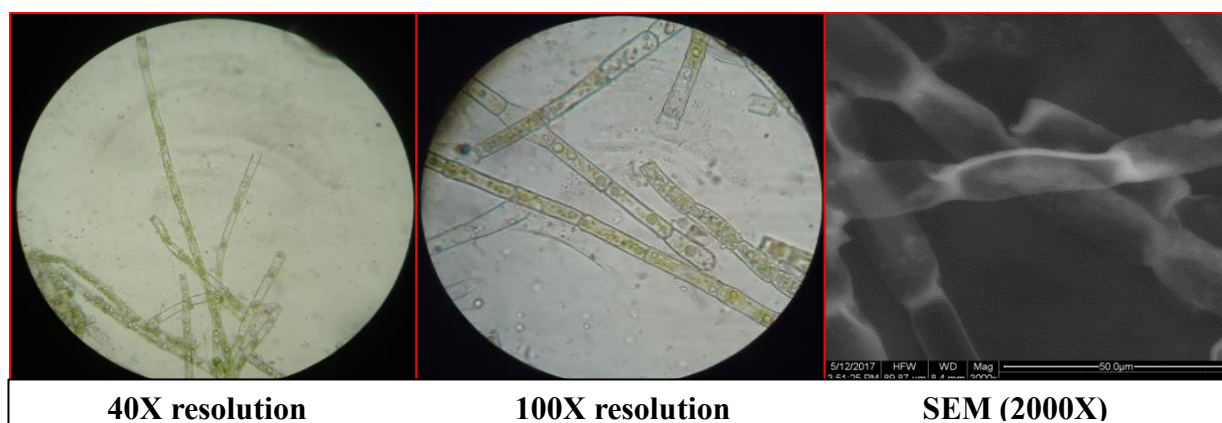


Figure 4.3: Microscopic identified *Oedogonium* sp .collected from Gadanahalli.

4.2.3 *Klebsormidium* sp.

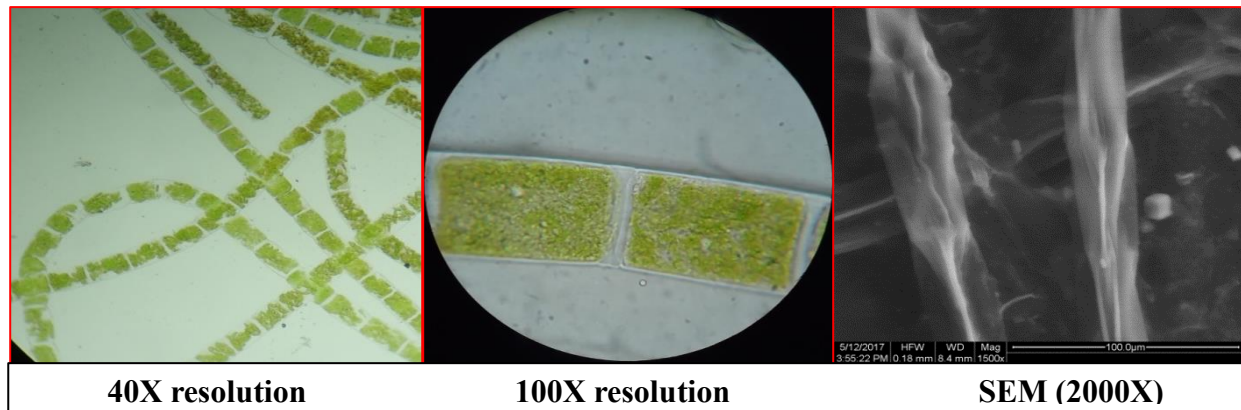


Figure 4.4: Microscopic identified *Klebsormidium*. Sp. collected from Ikola.

4.2.4 *Rhizoclonium* sp.

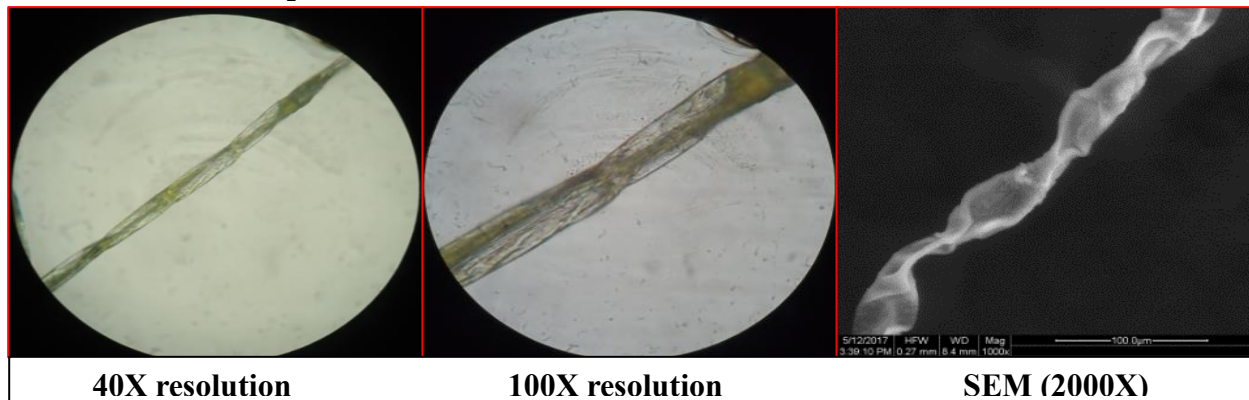


Figure 4.5: Microscopic identified *Rhizoclonium hieroglyphicum* collected from Dandiganahalli.

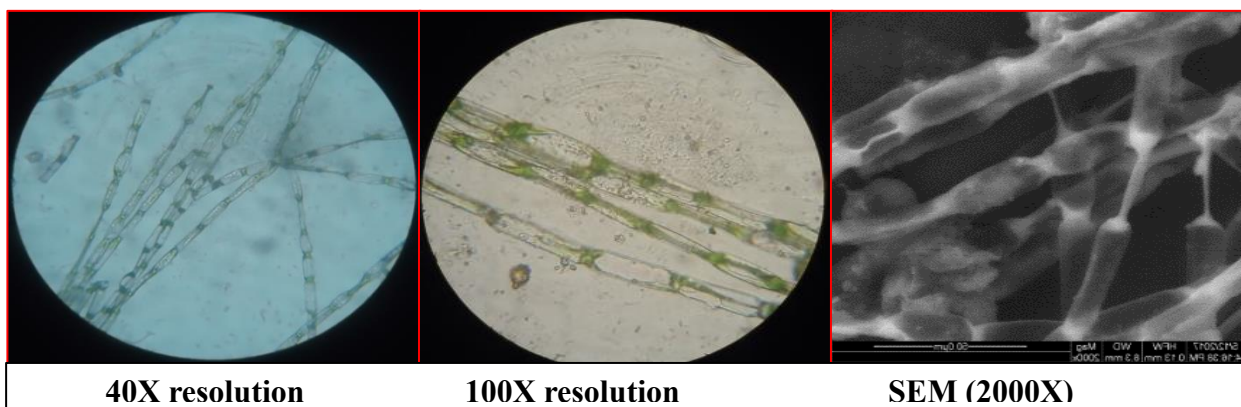


Figure 4.6: Microscopic identified *Rhizoclonium* sp. collected from Happanadka.

4.2.5 *Rhizoclonium hieroglyphicum*

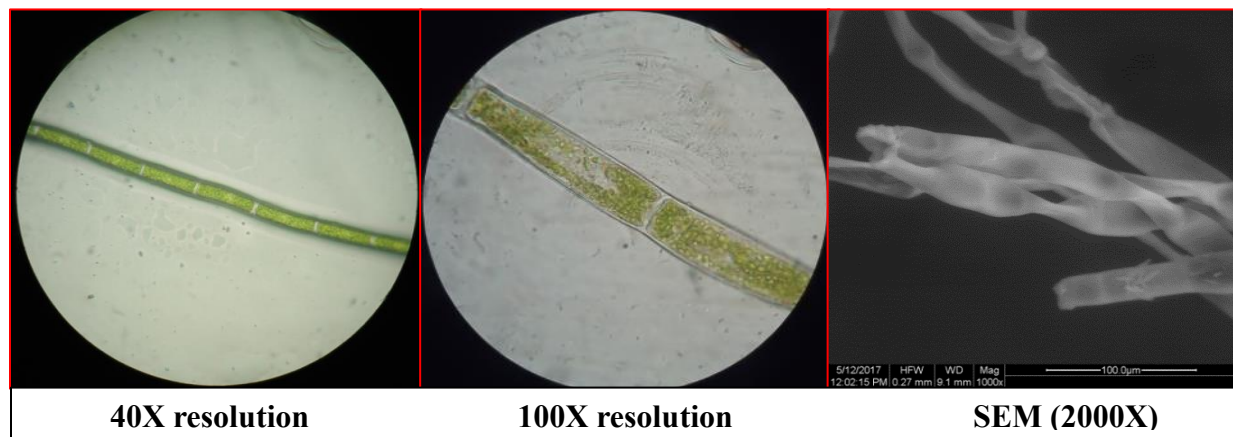


Figure 4.7: Microscopic identified *Rhizoclonium hieroglyphicum* collected from Kaggodu.

4.2.6 *Spirogyra* sp.

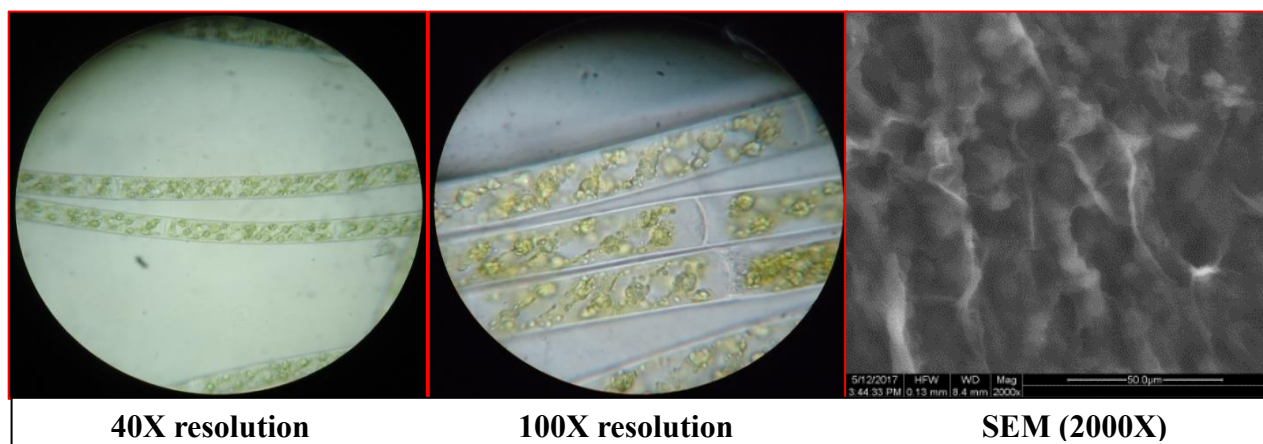


Figure 4.8: Microscopic identified *Spirogyra* sp. collected from Hoovnali (Hassan district)

4.3 Multiplication of terrestrial microalgae:

Collected algae were kept for multiplication in petri plates containing BB solid media shows good result. In that petri plates inoculated with *Cosmarium* sp., *Rhizoclonium* sp., *Rhizoclonium hieroglyphicum* and *Spirogyra* sp. tissues showed good response (**Figure 4.9**) and this culture was used for multiplication of terrestrial microalgae by batch culture and closed photobioreactor. In batch culture and closed photobioreactor microalgae samples were kept in BB liquid medium. In both culture methods *Rhizoclonium* sp. and *Rhizoclonium hieroglyphicum* collected from Dandiganahalli and Kaggodu respectively grown very well in a very short

duration. Growth kinetics of the terrestrial microalgae was estimated using batch culture from the 0th day to 50days interval under 600nm at spectrophotometer (**Figure 4.10**).

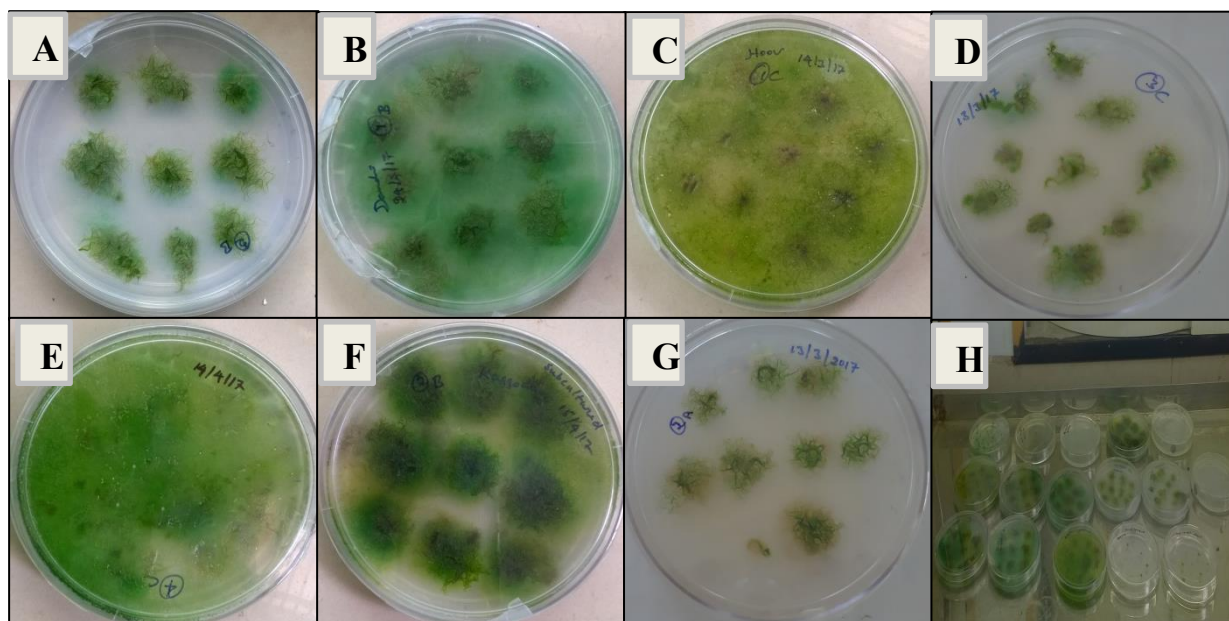


Figure 4.9: Multiplication of terrestrial microalgae from different location by petri plates. (A)Happanadka, (B) Dandiganahalli, (C) Hoovnalli, (D) Ikola, (E) ACH Campus, (F) Kaggodu, (G) Gadanahalli and (H) Culture petri plates at incubation chamber.

4.3.1 Growth Kinetic study:

In present study, *Rhizoclonium sp.* and *Rhizoclonium hieroglyphicum* was grew first, followed by *Spirogyra sp.*, *Cosmarium sp.*, *Oedogonium sp.* and *Klebsormidium sp.* appeared. In this study *Oedogonium sp.* and *Klebsormidium sp.* showed low growth compared to other species (**Figure 4.10**). From the seven weeks study terrestrial microalgae collected from different places such as *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.*, *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had initial optical density of 0.04 ± 0.003 , 0.06 ± 0.005 , 0.07 ± 0.005 , 0.05 ± 0.002 , 0.06 ± 0.003 , 0.07 ± 0.002 and 0.05 ± 0.006 , respectively and final optical density 2.01 ± 0.480 , 2.63 ± 0.008 , 2.41 ± 0.012 , 1.89 ± 0.019 , 2.43 ± 0.001 , 2.56 ± 0.150 and 1.85 ± 0.005 , respectively (**Table 4.2**). The results of present study are slightly variable with the study done by **Ismaiel et al. (2016)**, who observed 1.0 to 2.0 optical densities at different pH at 14days incubation of different species of microalgae. In current study growth rate slow because of the cells which were on culture flask were not in their growth phase.

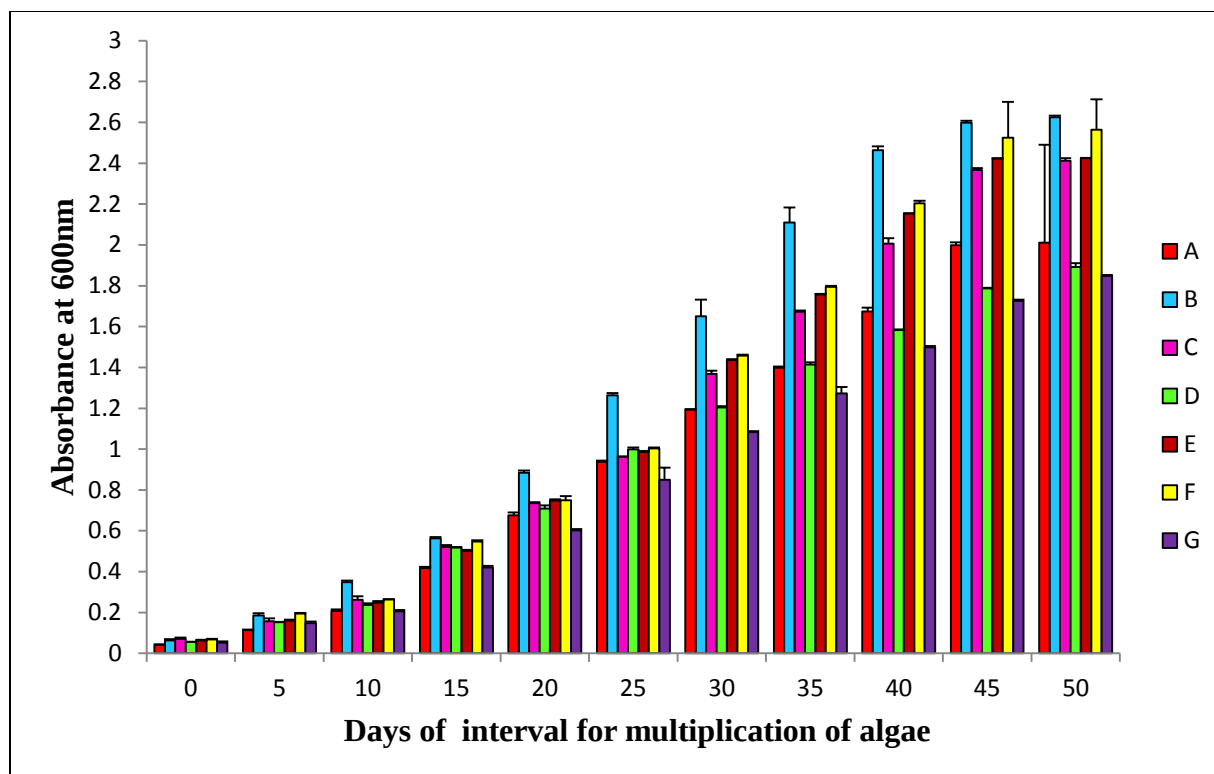


Figure 4.10: Optical density of batch cultured sample

- A** = Rhizoclonium sp. collected from Happanadka (Udupi district)
- B** = Rhizoclonium sp. collected from Dandiganahalli (Hassan district)
- C** = Spirogyra collected from Hoovnali (Hassan district)
- D** = Klebsormidium sp. Collected from Ikola (Kodagu district)
- E** = Cosmarium sp. collected from ACH campus (Hassan district)
- F** = Rhizoclonium hieroglyphicum collected from Kaggodu (Kodagu district)
- G** = Oedogonium sp. collected from Gadanahalli (Hassan district)

The maximum optical density observed in *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu) 2.63 ± 0.008 and 2.56 ± 0.150 at 600nm, which is indicator of highest biomass production. *Oedogonium sp.* collected from Gadanahalli (Hassan district) had lowest optical density of 1.85 ± 0.005 ; this may be because the cells which were on culture flask were not in their growth phase.

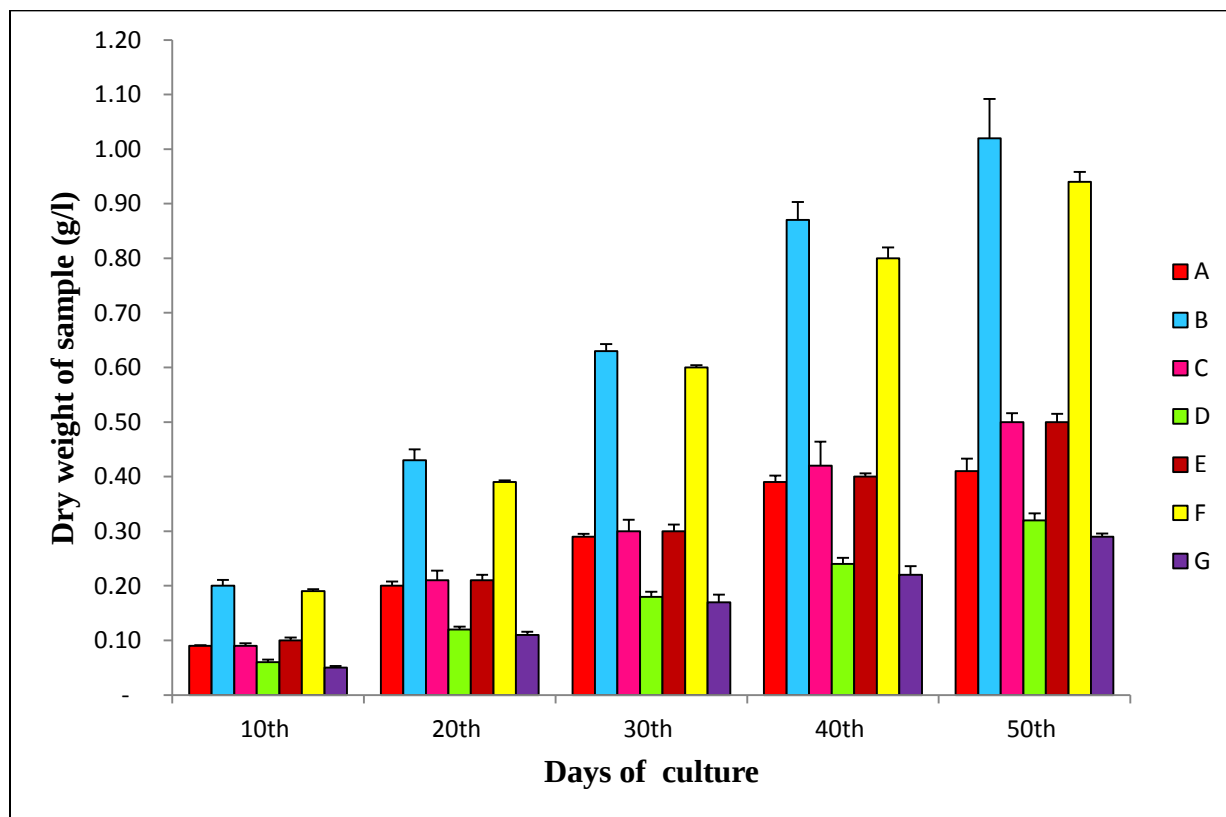


Figure 4.11: Dry weight of batch cultured sample

Dry weight of the terrestrial microalgae in batch culture was estimated. In this study *Rhizoclonium sp.* and *Rhizoclonium hieroglyphicum* had highest dry weight of 1.02 ± 0.072 g/and 0.94 ± 0.018 g/l respectively compared to others. *Oedogonium sp.* had minimum dry weight 0.29 ± 0.006 g/l (**Figure 4.11**). In this study, the resulted dry algal biomass harvested after incubation of 50days in the selected seven samples. The dry weight of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.*, *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) had initial(10th day) was observed as 0.09 ± 0.010 , 0.20 ± 0.011 , 0.09 ± 0.005 , 0.06 ± 0.005 , 0.10 ± 0.005 , 0.19 ± 0.004 and 0.05 ± 0.003 g/l, respectively and had final (50th day) dry weight of 0.41 ± 0.023 , 1.02 ± 0.072 , 0.50 ± 0.016 , 0.32 ± 0.013 , 0.50 ± 0.015 , 0.94 ± 0.018 and 0.29 ± 0.006 g/l, respectively was observed. *Rhizoclonium sp.* and *Rhizoclonium hieroglyphicum* showed good response in both batch and closed photobioreactor method. The result of present study slightly different from with the study done by Sethupathy *et al.* (2015), who observed that dry weight of

0.81 g/l, 0.89 g/l and 0.61 g/l in 24 days incubation. The variation was observed in dry weight because of their algae species was different from present studied species.

Table 4.3: Dry weight of batch cultured terrestrial microalgae (g/l)

Sample	10 th day	20 th day	30 th day	40 th day	50 th day
A	0.09 ± 0.010	0.20 ± 0.008	0.29 ± 0.005	0.39 ± 0.012	0.41 ± 0.023
B	0.20 ± 0.011	0.43 ± 0.020	0.63 ± 0.013	0.87 ± 0.033	1.02 ± 0.072
C	0.09 ± 0.005	0.21 ± 0.018	0.30 ± 0.021	0.42 ± 0.044	0.50 ± 0.016
D	0.06 ± 0.005	0.12 ± 0.005	0.18 ± 0.009	0.24 ± 0.011	0.32 ± 0.013
E	0.10 ± 0.005	0.21 ± 0.010	0.30 ± 0.012	0.40 ± 0.006	0.50 ± 0.015
F	0.19 ± 0.004	0.39 ± 0.003	0.60 ± 0.004	0.80 ± 0.020	0.94 ± 0.018
G	0.05 ± 0.003	0.11 ± 0.006	0.17 ± 0.014	0.22 ± 0.016	0.29 ± 0.006
F-test	S	S	S	S	S
S. Ed. (±)	0.003	0.006	0.007	0.010	0.018
C.D. (P=0.005)	0.008	0.017	0.022	0.030	0.054

All the values are MEAN ± SD of three replicates

Where,

A = *Rhizoclonium sp.* collected from Happanadka (Udupi district)

B = *Rhizoclonium sp.* collected from Dandiganahalli (Hassan district)

C = *Spirogyra* collected from Hoovnalli (Hassan district)

D = *Klebsormidium sp.* Collected from Ikola (Kodagu district)

E = *Cosmarium sp.* collected from ACH campus (Hassan district)

F = *Rhizoclonium hieroglyphicum* collected from Kaggodu (Kodagu district)

G = *Oedogonium sp.* collected from Gadanahalli (Hassan district)

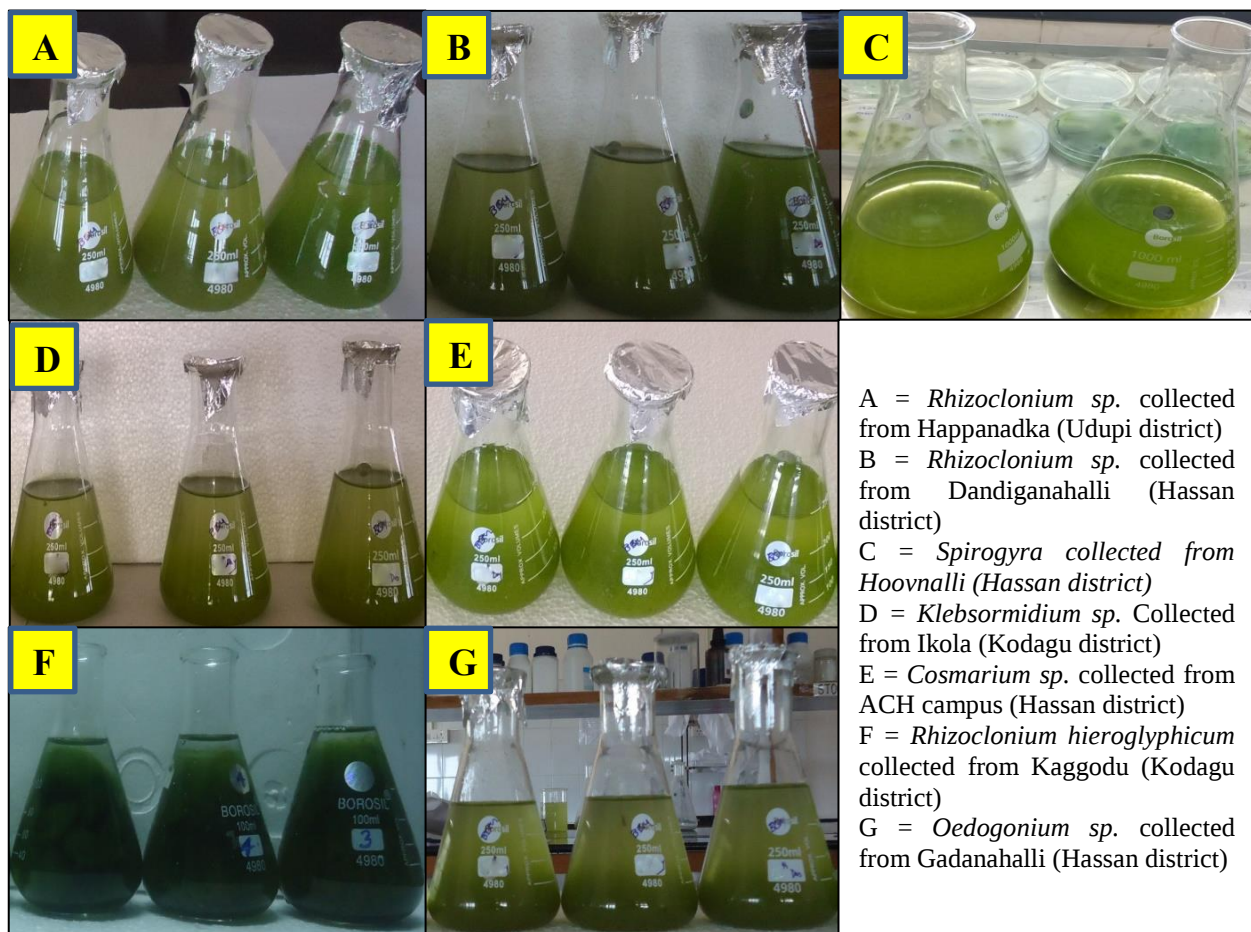


Figure 4.12: Multiplication of terrestrial microalgae by batch culture.



Figure 4.13: Mass multiplication of terrestrial microalgae by closed photobioreactor

(A) Happanadka, (B) Dandiganahalli, (C) Hoovnnalli, (D) Ikola, (E) ACH Campus, (F) Kaggodu and (G) Gadanahalli



Figure 4.14: Multiplication of terrestrial microalgae by open pond system

In the current study, *Rhizoclonium sp.* and *Rhizoclonium hieroglyphicum* collected from Dandiganahalli and Kaggodu respectively multiplied very fast in open pond system compared to other collected species. These two species grew very well in all four types of treated water viz, normal tap water, water with nutrient supplements, R.O. residual dispense water whose pH 8.4 and urban waste water. *Cosmarium sp.* showed good response to water with nutrients supplements and R.O. residual dispense water (**Figure 4.14**). In present study observed that open pond system was very cheap and a terrestrial microalga multiplies very fast but its main disadvantage was evaporation of water. So evaporation of water was controlled by using 50% shaded net during the entire period of study.

4.4 Biochemical characterization of selected species of terrestrial microalgae:

Biochemical characterization of terrestrial microalgae was done on the basis of chlorophyll, carbohydrates (total soluble sugars and total reducing sugars), protein and lipid contents. The results obtained are discussed below:

4.4.1 Chlorophyll content in terrestrial microalgae:

The results of chlorophyll content in terrestrial microalgae are presented in **Table 4.4**. The chlorophyll is the essential components for photosynthesis, and occurs in chloroplasts as green pigment in all photosynthetic plant tissues. The maximum chlorophyll a, b, c and total chlorophyll content was found in *Rhizoclonium hieroglyphicum* collected from Kaggodu (Kodagu district) as 14.82 ± 0.039 , 12.42 ± 0.007 , 7.11 ± 0.002 and 13.85 ± 0.087 mg/g tissue, respectively.

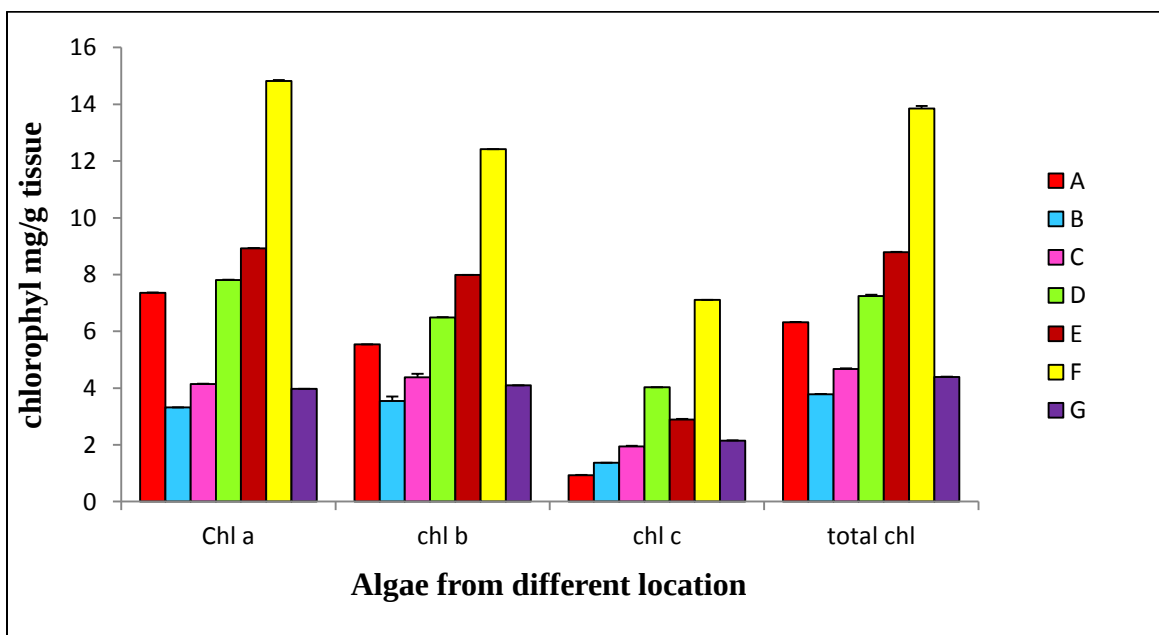


Figure 4.15: Chlorophyll content of terrestrial microalgae

The minimum chlorophyll a, b and total chlorophyll content was seen in *Rhizoclonium sp.* (Dandiganahalli) as 3.31 ± 0.002 , 3.55 ± 0.151 and 3.78 ± 0.011 mg/g tissue, respectively. The minimum chlorophyll c of 0.93 ± 0.014 mg/g tissue was observed in *Rhizoclonium sp.* (Happanadka). In current study chlorophyll a, b, c and total chlorophyll content in *Rhizoclonium hieroglyphicum* has 77.63, 71.41, 80.78 and 72.68% more than *Rhizoclonium sp.* (Happanadka). The results obtained in the present study were statistically significant. The results of present study are in accordance with the similar study done by **Benemann et al. (1978)** and **Ismail et al. (2016)** who observed 10.6mg/g of Chl a.

Table 4.4: Chlorophyll content of terrestrial microalgae

Sample	Chlorophyll a (mg/g tissue)	Chlorophyll b (mg/g tissue)	Chlorophyll c (mg/g tissue)	Total chlorophyll (mg/g tissue)
A	7.36 ± 0.008	5.54 ± 0.008	0.93 ± 0.014	6.32 ± 0.005
B	3.31 ± 0.002	3.55 ± 0.151	1.37 ± 0.004	3.78 ± 0.011
C	4.14 ± 0.011	4.38 ± 0.122	1.94 ± 0.019	4.68 ± 0.014
D	7.81 ± 0.011	6.49 ± 0.011	4.03 ± 0.005	7.25 ± 0.045
E	8.93 ± 0.011	7.98 ± 0.003	2.89 ± 0.030	8.78 ± 0.016
F	14.82 ± 0.039	12.42 ± 0.007	7.11 ± 0.002	13.85 ± 0.087
G	3.97 ± 0.005	4.10 ± 0.001	2.15 ± 0.003	4.39 ± 0.010
F-test	S	S	S	S
S. Ed. ($\pm$)	0.010	0.038	0.009	0.020
C. D. (P = 0.05)	0.032	0.118	0.028	0.062

All the values are MEAN $\pm$ SD of three replicates

4.4.2 Total soluble sugar content in terrestrial microalgae:

Total soluble sugar quantitatively determined by Anthrone method because of its sensitivity towards sugar. Terrestrial microalgae collected from different places such as *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) had contain total soluble sugar 44.81 ± 0.16 , 38.18 ± 0.95 , 37.10 ± 0.11 , 34.68 ± 0.04 , 34.79 ± 0.12 , 42.16 ± 0.04 and $35.36 \pm 0.10\%$, respectively (**Table 4.5**). The results obtained in the present study were statistically significant. Maximum percentage of TSS obtained in the *Rhizoclonium sp.* collected from the river of Happanadka

(Udupi district) as $44.81 \pm 0.16\%$ and minimum observed in the *Klebsormidium sp.* $34.68 \pm 0.04\%$ (**Figure 4.17**). These total soluble sugars are hydrolyzed by 2.5N HCl, which was helped in saccharification and these hydrolyzed sugar were used as the sugar source for yeast (*Saccharomyces cerevisiae*) which breaking the sugar contents in the algal residue to produce ethanol. The results of current study are in accordance with the similar study done by **Lalitesh et al. (2017)**.

4.4.3 Total reducing sugar content in terrestrial microalgae:

In the present study reducing sugar were estimated by DNS method. Terrestrial microalgae collected such as *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) had 24.60 ± 0.27 , 24.40 ± 0.40 , 24.10 ± 0.36 , 23.80 ± 0.92 , 25.30 ± 0.60 , 23.90 ± 0.17 and $24.40 \pm 0.27\%$ of total reducing sugar, respectively (**Table 4.5**). Maximum percentage of TRS obtained in the *Cosmarium sp.* $25.30 \pm 0.60\%$ and minimum observed in the *Klebsormidium sp.* $23.80 \pm 0.92\%$ (**Figure 4.17**). These reducing sugars are monosaccharides and disaccharides which were easily breakdown to produce ethanol by yeast. The results of present study are in accordance with the similar study done by **Lalitesh et al. (2017)** and the results obtained are statistically significant.

4.4.4 Protein content in terrestrial microalgae:

In the current study total protein were estimated by Lowry method. Terrestrial microalgae *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.*, *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had 9.93 ± 0.03 , 22.24 ± 0.03 , 6.64 ± 0.03 , 5.12 ± 0.16 , 13.08 ± 0.05 , 19.51 ± 0.04 and 5.73 ± 0.02 mg/g of protein, respectively (**Table 4.5**). Maximum protein content was observed in *Rhizoclonium sp.* collected from pond of Dandiganahalli (Hassan district) as 22.24 ± 0.03 and minimum observed in the *Klebsormidium sp.* 5.12 ± 0.16 mg/g (**Figure 4.17**). SDS- PAGE were conducted for these proteins and all terrestrial microalgae bands are observed in 41kDa, 30kDa and 22kDa region in the gel it indicates the terrestrial microalgae proteins as 41kDa, 30kDa and 22kDa of molecular weight (**Figure 4.16**). The results obtained in present study are statistically significant. The result of present study is in accordance with the similar study done by **Peter et al. (2010)**. According to **Peter et al. (2010)** higher protein content increases the lipid content in the microalgae and they observed 27-48% of protein in various species of microalgae.

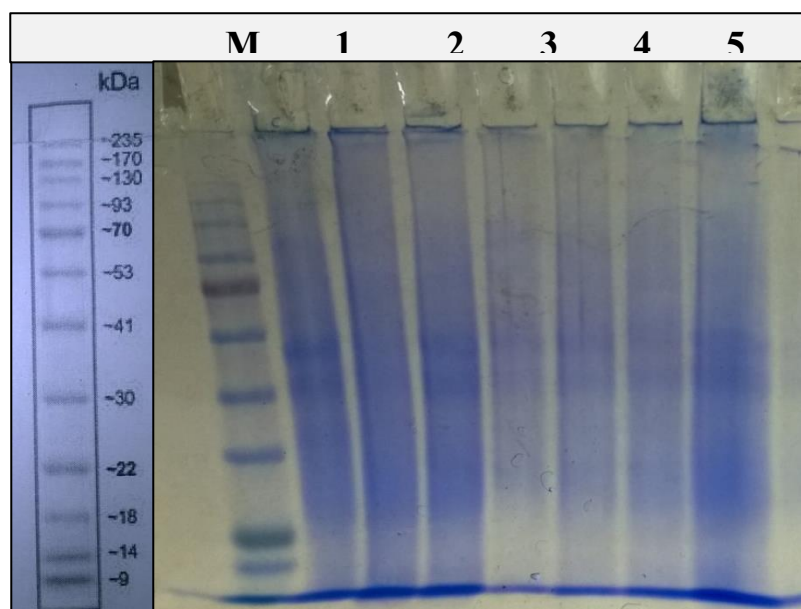


Figure4.16: SDS-PAGE for protein content of terrestrial microalgae

Where M. Marker (9-235kDa), 1,2,3,4,5,6 and 7 indicate the sample in that well are *Rhizoclonium sp.* (Hapannadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirgyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli), respectively.

Table 4.5: Biochemical characterization of terrestrial microalgae

SAMPLES	TSS (%)	TRS (%)	LIPID (%)	PROTEIN(mg/g)
A	44.81 ± 0.16	24.60 ± 0.27	31.25 ± 1.52	9.93 ± 0.03
B	38.18 ± 0.95	24.40 ± 0.40	43.00 ± 2.18	22.24 ± 0.03
C	37.10 ± 0.11	24.10 ± 0.36	32.75 ± 1.53	6.64 ± 0.03
D	34.68 ± 0.04	23.80 ± 0.92	31.75 ± 1.52	5.12 ± 0.16
E	34.79 ± 0.12	25.30 ± 0.60	31.50 ± 3.12	13.08 ± 0.05
F	42.16 ± 0.04	23.90 ± 0.17	41.25 ± 1.09	19.51 ± 0.04
G	35.36 ± 0.10	24.40 ± 0.27	36.00 ± 0.50	5.73 ± 0.02
F- test	S	S	S	S
S.Ed. (±)	0.210	0.410	1.050	0.040
C.D. (P=0.05)	0.648	1.268	3.230	0.119

All the values are MEAN ± SD of three replicates

4.4.5 Lipid content in terrestrial microalgae:

The results of lipid contents in terrestrial microalgae are presented in **Table 4.5**. In the present study the seven terrestrial microalgae (A, B, C, D, E, F and G) had lipid content of 31.25 ± 1.52 , 43.00 ± 2.18 , 32.75 ± 1.53 , 31.75 ± 1.52 , 31.50 ± 3.12 , 41.25 ± 1.09 and $36.00 \pm 0.50\%$, respectively. In this highest lipid content were found in *Rhizoclonium sp.* (Dandiganahalli) $43.00 \pm 2.18\%$, followed by *Rhizoclonium hieroglyphicum* (Kaggodu) $41.25 \pm 1.09\%$ and $31.25 \pm 1.52\%$ of lowest lipid content found in a *Rhizoclonium sp.* (Happanadka).

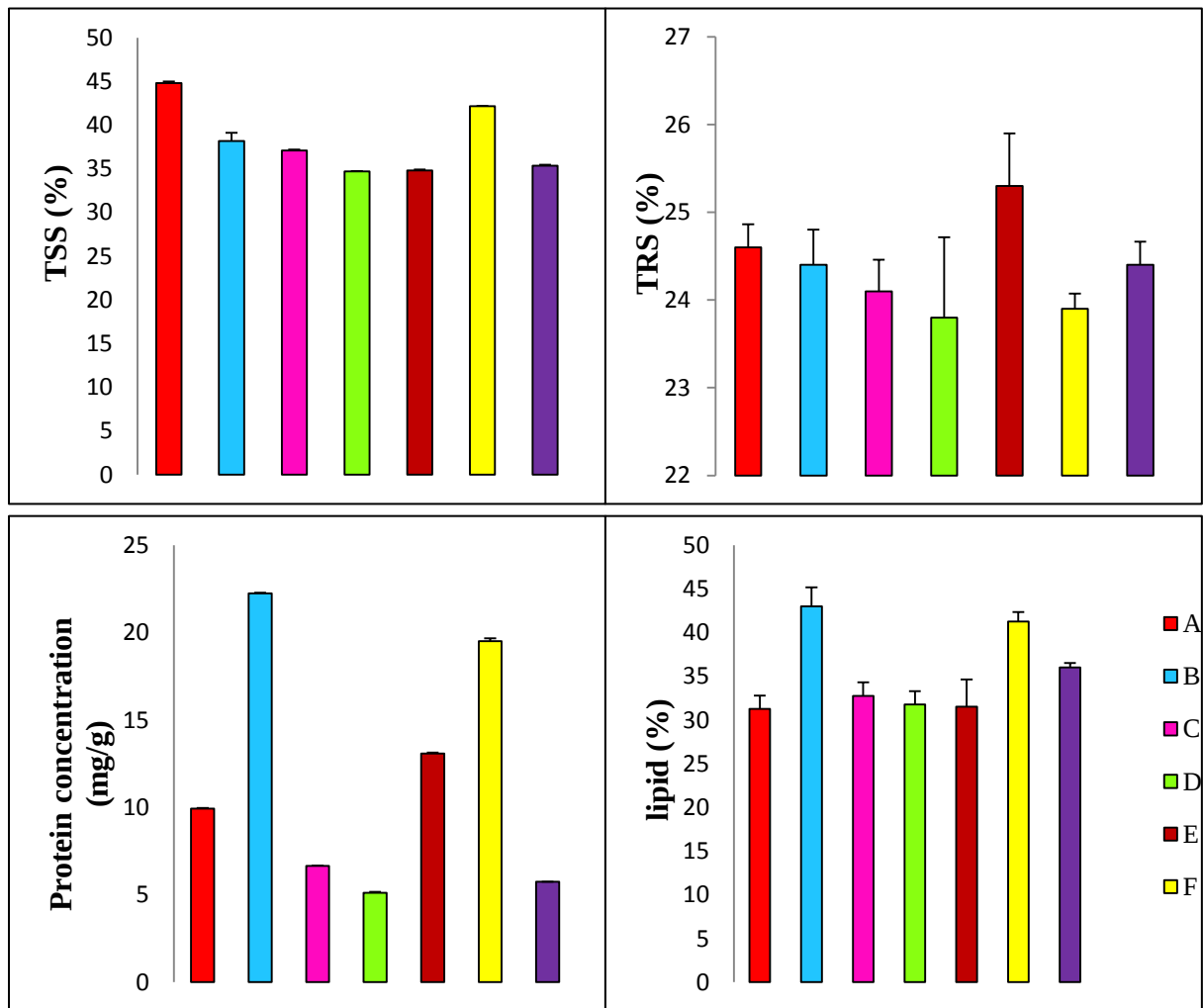


Figure 4.17: Biochemical characterization of Terrestrial microalgae

The results of lipid content in present study are in accordance with the similar study done by **Medipally et al. (2015)** who noted microalgae with low oil content was 30%, microalgae with medium oil content was 50% and microalgae with high oil content was 70%. Lipid content of present studied terrestrial microalgae had variable because of different species used for

estimation and also the biodiversity of the species, habitat and locations (**Metting, 1996**). The results obtained in present study were statistically significant. The similar study also done by **Abishek et al. (2014)** who obtained 15-55% of lipid content on different species of green and brown algae. **Brennan and Owende (2010)** who noticed 42-57.8% of lipid content on different species of microalgae.

4.5 Transesterification of microalgal oil for biodiesel production:

Microalgal biomass is a potential feedstock for biofuel production. The result of production of biodiesel and glycerin from the algal oil were observed in the **Table 4.6**. In current study terrestrial microalgae *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.*, *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* produced biodiesel 72.50 ± 1.73 , 75.00 ± 1.50 , 80.00 ± 1.80 , 65.00 ± 1.73 , 80.00 ± 1.32 , 70.00 ± 1.73 and $65.00 \pm 0.87\%$ (**Table 4.6**), from the algal oil of 31.25 ± 1.52 , 43.00 ± 2.18 , 32.75 ± 1.53 , 31.75 ± 1.52 , 31.50 ± 3.12 , 41.25 ± 1.09 and $36.00 \pm 0.50\%$, respectively (**Table 4.5**). The results obtained in present study were statistically significant.

Table 4.6: Production of Biodiesel and Glycerin from terrestrial microalgae oil

Samples	Diesel (%)	Glycerin (%)
A	72.50 ± 1.73	25.30 ± 0.36
B	75.00 ± 1.50	20.00 ± 1.76
C	80.00 ± 1.80	18.00 ± 0.70
D	65.00 ± 1.73	28.00 ± 0.36
E	80.00 ± 1.32	15.00 ± 0.25
F	70.00 ± 1.73	24.00 ± 0.80
G	65.00 ± 0.87	25.00 ± 0.36
F-test	S	S
S. Ed. ($\pm$)	0.86	0.44
C.D. (P=0.05)	2.65	1.34

All the values are MEAN $\pm$ SD of three replicates

In this study maximum content of 80.00 ± 1.32 and $80.00 \pm 1.80\%$ biodiesel was recovered from the *Spirogyra sp.* and *Cosmarium sp.* which as algal oil of 32.75 ± 1.53 and $31.50 \pm 3.12\%$, respectively. Along with biodiesel glycerin also produced, maximum glycerin produced in *Klebsormidium sp.* (Ikola) as $28.00 \pm 0.36\%$ from the algal oil of $31.75 \pm 1.52\%$ and minimum in *Cosmarium sp.* (ACH campus) as $15.00 \pm 0.25\%$ from the algal oil of $31.50 \pm 3.12\%$ (**Figure 4.18**). The result of current study is in accordance with the similar study done by **Shalaby (2011)**.

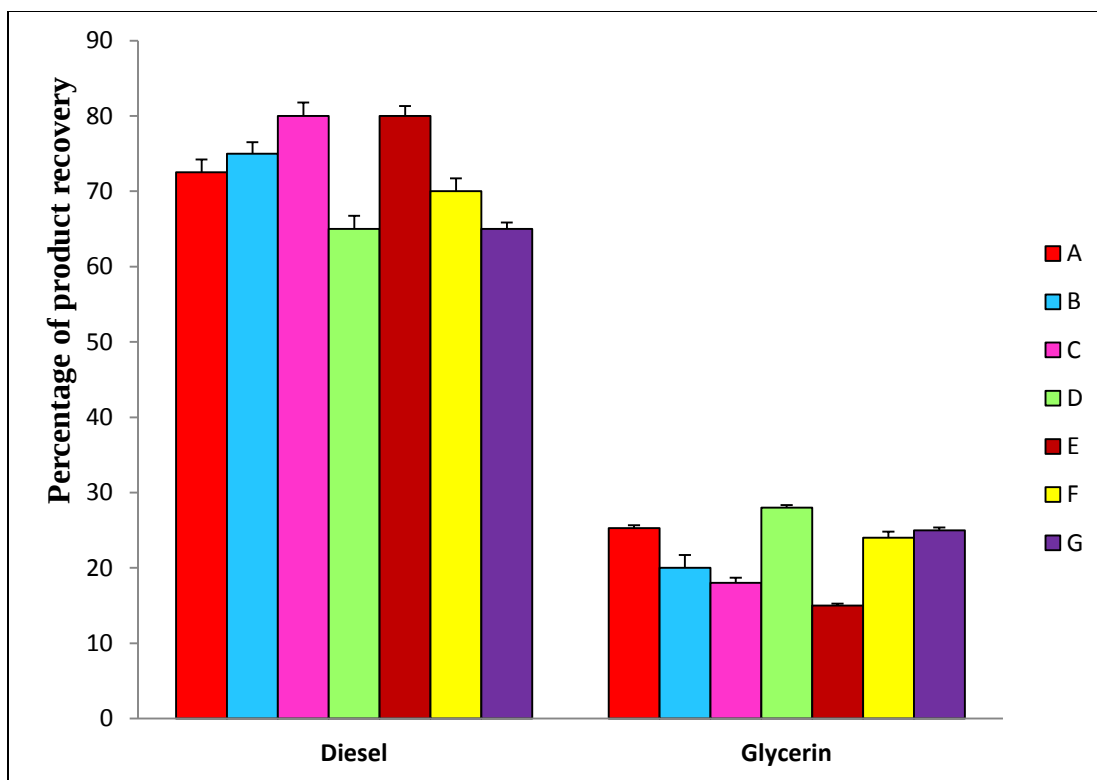


Figure 4.18: Production of Biodiesel and Glycerin from terrestrial microalgae oil

4.6: Bioethanol production by fermentation of Terrestrial microalgae:

In the current study bioethanol were produced by fermenting the defatted algal residues and standardized the ethanol production on the basis of TSS, TRS, protein, pH, organic acids, microbial load, °brix and alcohol content of anaerobic fermentation setup of terrestrial microalgae in duration of 21 days showed below.

4.6.1: Total soluble sugars:

The results of total soluble sugars were estimated from fermentation setup of terrestrial microalgae for every 3days of interval from 0th day to 21st days is presented in **Table 4.7**. In present study the total soluble sugar decreased, the decreased percentage difference between 0th day and 9th day of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.*, *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had 53.08, 59.06, 51.03, 55.84, 49.98, 44.75 and 46.45%, respectively.

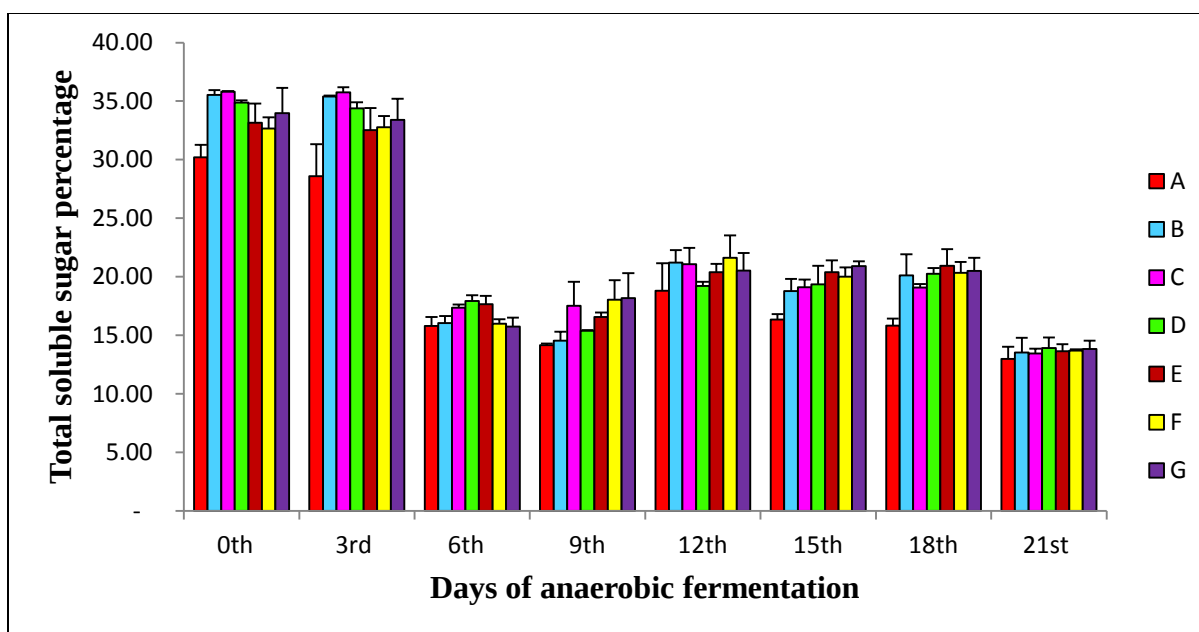


Figure 4.19: Total soluble sugars estimated from the fermentation setup of terrestrial microalgae.

The maximum decreases of sugar were found in *Rhizoclonium sp.* (Dandiganahalli), where $35.54 \pm 0.4\%$ of total soluble sugar on 0th day decreased to $14.55 \pm 0.75\%$ on 9th day. This decreased percentage difference of 0th day and 9th day was 59.06%. It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period to ethanol. But after 12th to 18th days, TSS percentage was increased because of releasing sugar content from algal biomass inside fermentation set up and it is known as secondary fermentation. Yeast (*Saccharomyces cerevisiae*) utilized these sugar content so on 21st days total soluble sugar content were decreased in all present studied sample (**Figure 4.19**) and decreased percentage difference between 0th day and 21st day was 56.99, 62.43, 61.93, 60.11, 58.82, 58.03 and 59.29%, respectively. On 21st day also *Rhizoclonium sp.* (Dandiganahalli) has decreased total soluble sugar to $13.53 \pm 1.25\%$, giving 62.43 % difference in this period. The values obtained in present study except 12th and 21st day all are statistically significant. These microalgae had high carbohydrate content and could be efficiently saccharified to monosugars using sulfuric acid, which could serve as good substrates for the fermentative production of organic acids. Besides, the protein, nitrogen and ash in the hydrolysate might be favorable ingredients supporting fermentation. These total soluble sugars after hydrolysis can act as a substrate for fermentation. The results of present study are in accordance with the similar study done by **Lalitesh et al. (2017)**, who also observed decreased sugar content during fermentation on different species of microalgae.

4.6.2 Total reducing sugar:

The results of total reducing sugars were estimated from fermentation setup of terrestrial microalgae for every 3 days of interval from 0th day to 21st day is presented in **Table 4.8**. In the current study the total reducing sugar decreased, the decreased percentage difference between 0th day and 21st day of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) were 25.84, 30.70, 30.71, 32.27, 34.31, 33.35 and 38.52%, respectively. The maximum reducing sugar decreased percentage difference were observed in *Oedogonium sp.* (Gadanahalli) 38.52%, it as reducing sugar content on 0th day $24.40 \pm 0.10\%$ it was decreased gradually and on 21st day of fermentation reducing sugar concentration $15.00 \pm 1.36\%$ were observed (**Figure 4.20**). The values obtained on present study statistically significant except 6th, 15th and 18th day. The sugar content in all species of present study decreased gradually from 0th day to 21st days due the utilization of sugar by the yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period and produced the ethanol. The results of present study are in accordance with the similar study done by **Lalitesh et al. (2017)**, who also observed decreased sugar content during fermentation on different species of microalgae.

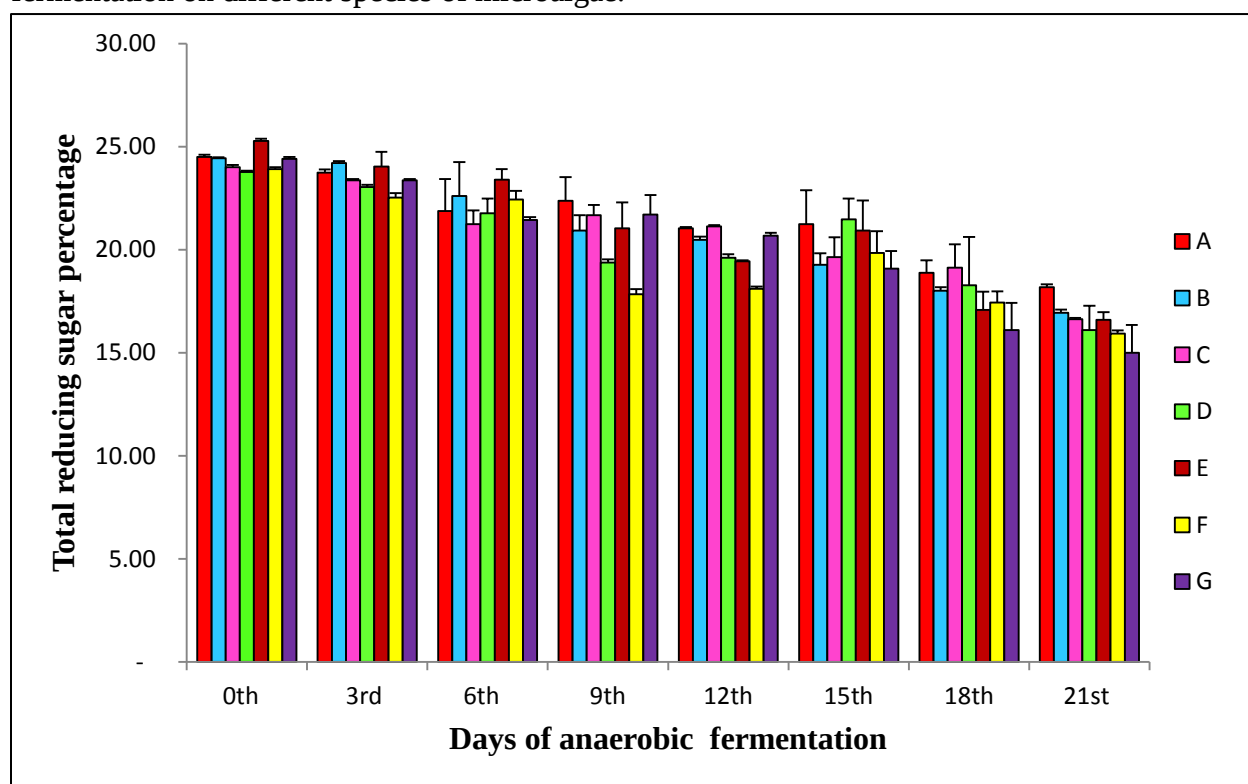


Figure 4.20: Total reducing sugars estimated from the fermentation setup of terrestrial microalgae

4.6.3: Total protein:

The results of total proteins were estimated from fermentation setup of terrestrial microalgae for every 3 days of interval from 0th day to 21st days is presented in **Table 4.9**. In present study the total proteins are gradually increased, the increased percentage difference between 0th day and 21st day of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* are 7.21, 1.01, 33.79, 36.71, 46.37, 33.06 and 3.14%, respectively. *Klebsormidium sp.* had maximum increased from 0th day to 21st day was 2.07 ± 0.53 to 2.83 ± 0.80 mg/g the increased percentage difference was 46.37%. During fermentation protein content were increased due to decreased sugar content. In present study protein content of *Klebsormidium sp.* were gradually increased from 3rd day to 21st days during anaerobic fermentation period are 2.07 ± 0.53 , 1.55 ± 0.08 , 1.62 ± 0.06 , 1.72 ± 0.11 , 1.79 ± 0.17 , 1.95 ± 0.31 and 2.22 ± 0.50 mg/g, respectively (**Figure 4.21**).

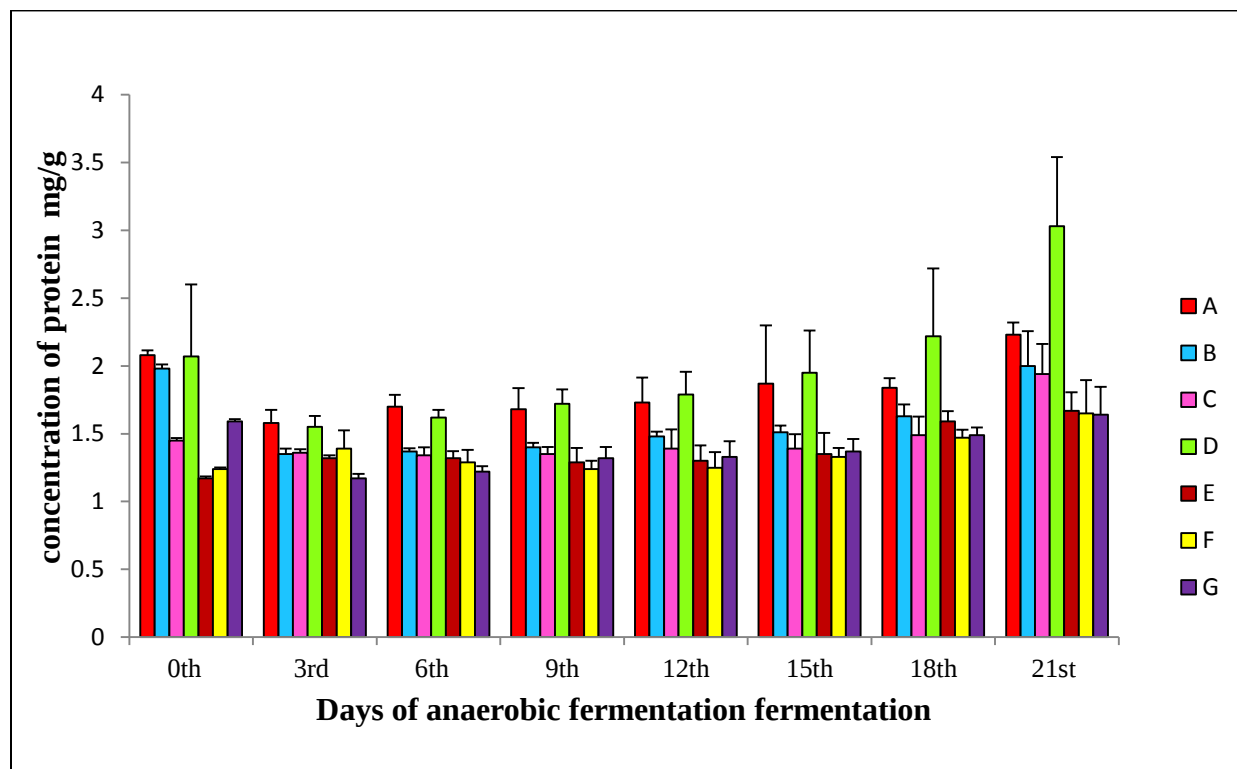


Figure 4.21: Protein estimated from the fermentation setup of terrestrial microalgae

The value observed in present study was statistically significant. The result of present study is in accordance with the similar study done by **Peter et al. (2010)**. According to whom higher protein content increases production of other byproducts like enzymes, nucleic acids and organic acids which helps production of ethanol.

4.6.4 pH:

The results of the pH were estimated from fermentation setup of terrestrial microalgae for every 3 days of interval from 0th day to 21st days is presented in **Table 4.10**. In present study the pH were decreased, the decreased percentage difference between 0th day and 9th day of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.*, *Cosmarium sp.*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp.* had 16.24, 16.13, 16.53, 15.43, 5.99, 9.17 and 18.72%, respectively. The maximum decrease of sugar were found in *Oedogonium sp.* 7.80 ± 0.20 of pH on 0th day and it decreased to 6.95 ± 0.05 pH on 9th day, the decreased percentage difference of 0th day and 9th day was 18.72%. It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period which increases the microbial load and produced the ethanol. But after 12th to 18th day's pH percentages were increased, it because of increasing TSS inside anaerobic fermentation set up.

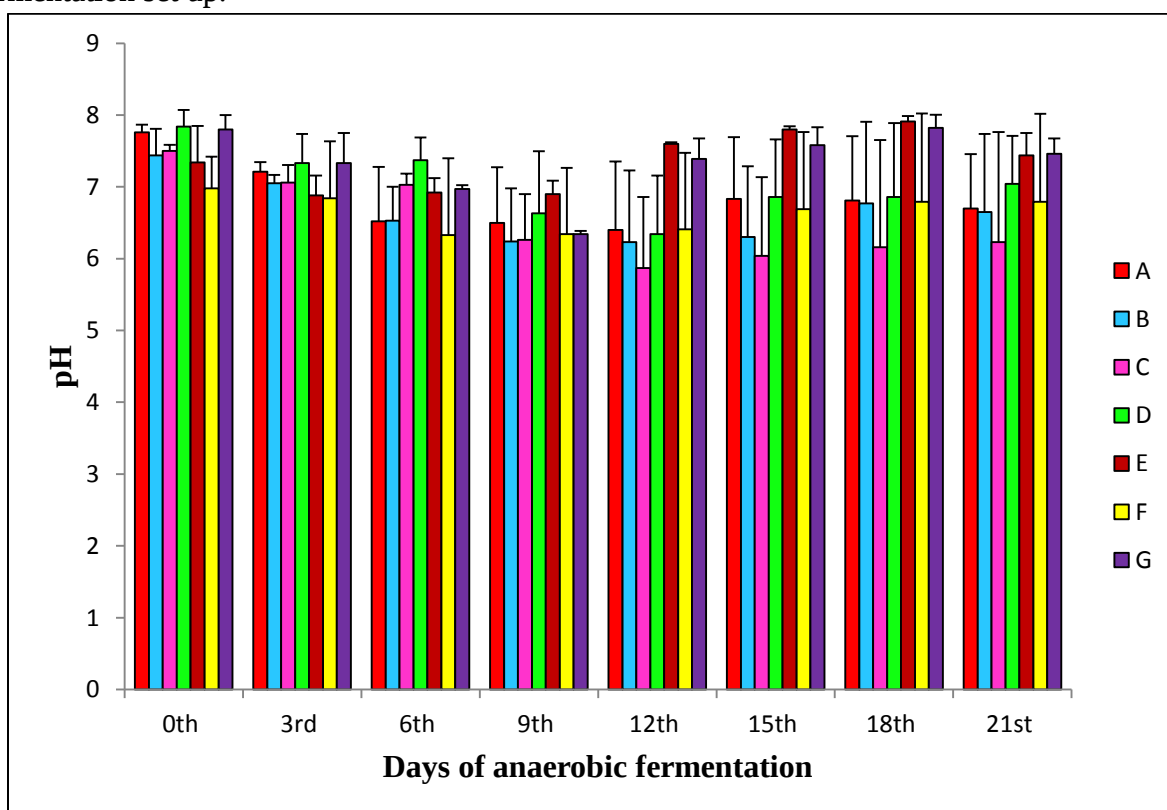


Figure 4.22: pH estimated from the fermentation setup of terrestrial microalgae

The pH of samples on 21st days decreases (**Figure 4.22**) because of yeast (*Saccharomyces cerevisiae*) utilized these sugar content so total soluble sugar content was decreased in all present studied sample (**Figure 4.19**) and increased the microbial growth. Decreased percentage difference between 0th day and 21st day was 13.66, 10.62, 16.93, 10.20, 1.36, 2.7% and 4.36%, respectively. On 21st day *Spirogyra sp.* had decreased the pH to 6.23 ± 1.53 , the decreased

percentage difference of 0th day and 21st day was 16.93%. The rate of ethanol production maximum at pH 6.79 was observed. pH plays an important role in the growth and adaptation of microorganism. The results obtained in present study were statistically not significant. The results of present study are in accordance with the similar study done by **Lalitesh et al. (2017)**, in their study microorganism growth increased below pH 7 and maximum ethanol produced at pH 6. Present study was slightly varies from **Lalitesh et al. (2017)** study because of their species was differ from present studied species.

4.6.5 Microbial load:

The results of microbial load were estimated from fermentation setup of terrestrial microalgae for every 3days of interval from 0th day to 21st days is presented in **Table 4.11**. In present study the microbial load was increased. The 0th day optical densities of present studied seven terrestrial microalgae are 0.39 ± 0.01 , 0.40 ± 0.02 , 0.36 ± 0.04 , 0.38 ± 0.01 , 0.38 ± 0.03 , 0.38 ± 0.02 and 0.39 ± 0.01 , respectively. On 9th day optical density was increased to 1.45 ± 0.03 , 1.46 ± 0.01 , 1.74 ± 0.11 , 1.16 ± 0.10 , 0.77 ± 0.04 , 1.75 ± 0.16 and 0.68 ± 0.08 , respectively.

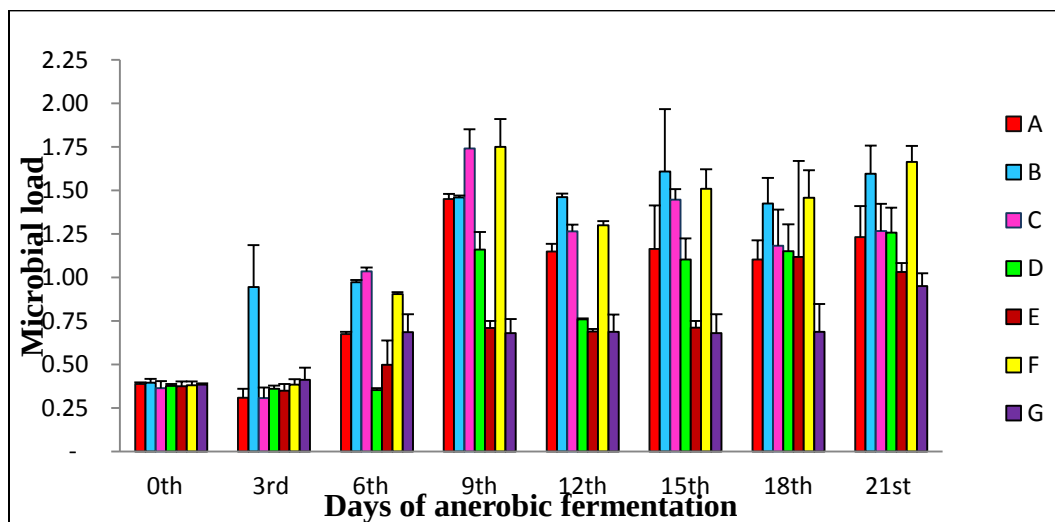


Figure 4.23: Microbial load estimated from the fermentation setup of terrestrial microalgae

The maximum increase of microbial load were found in *Spirogyra sp.* It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period which increases the microbial load and decreases the pH. Microbes were multiplies very quickly at acidic pH of 5.5 to 6.8. The environmental factors influence the growth of a microorganism such as substrate, temperature and pH. In present study the pH of 6.2 to 6.9 was observed on 9th day of fermentation (**Table 4.10**) so more microbial growth were observed on that period. But after 12th to 18th days microbial load was decreased, it because of increasing TSS inside anaerobic fermentation set up (**Figure 4.19**).

The microbial load of samples on 21st days increases because of yeast (*Saccharomyces cerevisiae*) utilized these sugar content so total soluble sugar content were decreased in all present studied sample (**Table 4.7**) and increased the microbial growth. Microbial load was increased on 21st days, microbe's optical growth were 1.23 ± 0.18 , 1.59 ± 0.6 , 1.27 ± 0.16 , 1.26 ± 0.14 , 1.03 ± 0.05 , 1.66 ± 0.09 and 0.95 ± 0.07 , respectively (**Figure 4.23**). The rate of ethanol production maximum at pH 6.79 was observed. The results obtained in the present study were statistically significant except 0th day. pH plays an important role in the growth and adaptation of microorganism. The results of present study are in accordance with the similar study done by **Lalitesh et al. (2017)**, who observed microorganism growth increased between pH 5.5-6.5. Present study was slightly different from **Lalitesh et al. (2017)**.

4.6.6 Alcohol content:

The results of total alcohol percentage were estimated from fermentation setup of terrestrial microalgae for every 3 days of interval from 0th day to 21st days is presented in **Table 4.12**. In present study the alcohol contents were high on 6th and 21st day. The alcohol quantity observed on 6th day of *Rhizoclonium sp.* (Happanadka), *Rhizoclonium sp.* (Dandiganahalli), *Spirogyra sp.* (Hoovnalli), *Klebsormidium sp.* (Ikola), *Cosmarium sp.* (ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.* (Gadanahalli) are 100.49, 109.15, 104.57, 100.32, 100.81, 111.11 and 106.37mg/ml, respectively. The maximum alcohol quantity was observed on 21st day of present studied samples are 115.68, 119.28, 115.03, 115.35, 117.32, 123.52 and 117.15mg/ml, respectively (**Figure 4.24**). The percentages of alcohol content on 21st day are 57.84 ± 0.65 , 59.64 ± 0.79 , 57.51 ± 0.99 , 57.67 ± 0.51 , 58.60 ± 0.37 , 61.76 ± 1.12 and $58.57 \pm 0.49\%$, respectively.

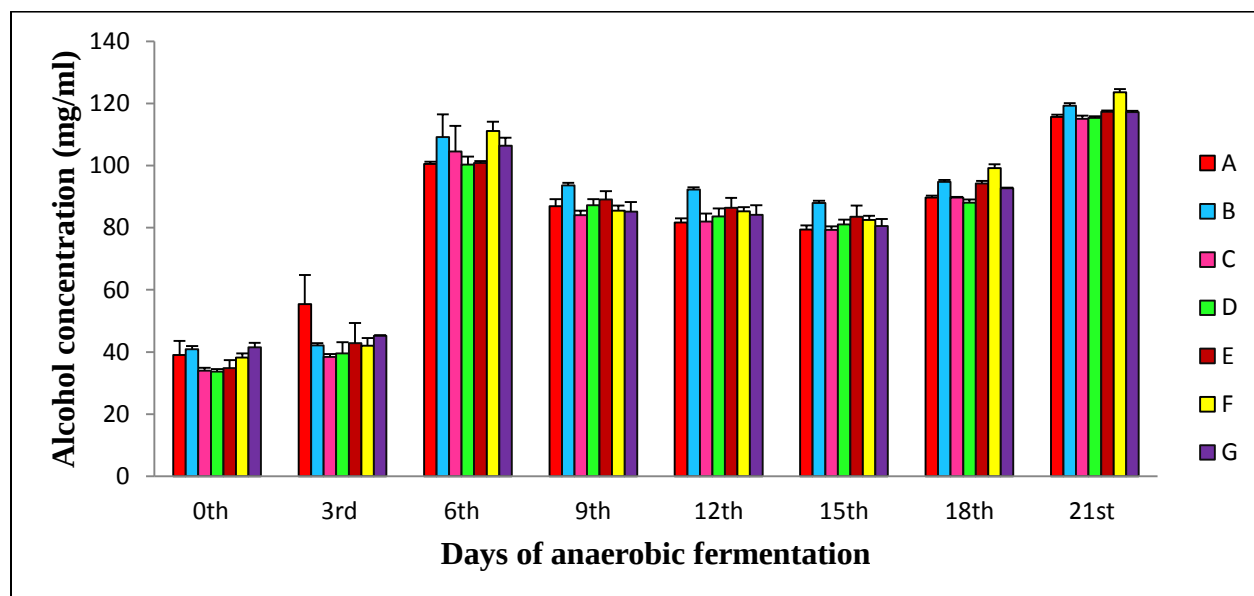


Figure 4.24: Alcohol estimated from the fermentation setup of terrestrial microalgae.

The maximum alcohol percentage was founded on *Rhizoclonium hieroglyphicum* (Kaggodu) and *Rhizoclonium sp.* (Dandiganahalli) are 61.76 ± 1.12 and $59.64 \pm 0.79\%$ (**Table 4.14**). It was mainly because of yeast (*Saccharomyces cerevisiae*) which breakdown the sugar content during the fermentation period and produced the ethanol. During the period of fermentation of 6th and 21st day carbohydrates content in fermentation setup decreases and microbial load increases, it is also a one reason for increased percentage of alcohol on 6th and 21st day. But after 12th to 18th days alcohol percentage were decreases, because of decreasing microbial load due to increase in pH inside fermentation set up. Yeast (*Saccharomyces cerevisiae*) utilizes the sugar content present in the algal biomass residues during fermentation period so on 21st days total soluble sugar content were decreased in all present studied samples (**Figure 4.19**) which were advantage to produce more quantity of alcohol. Sugar consumption held at the time of exponential phase in fermentation was observed. Microalgal sugars were consumed and simultaneously ethanol was produced. At the period of bio-ethanol fermentation, the concentration of the reducing sugar decreased with respect to days, and the concentration of bio-ethanol further increased gradually, clearly indicating that present studied seven samples were used as fermentation sugars. The results of present study are in accordance with the similar study done by Lalitesh *et al.* (2017).

4.8 Effects of terrestrial microalgae on treated water:

In current study effect of terrestrial microalgae on treated water on the basis of Chloride content, TDS and pH in duration of 60days days showed below. In present study the iron content in treated water of normal tap water (**T₁**), water with nutrients (**T₂**), R.O. residual dispense waste (**T₃**) and urban waste water (**T₄**) up to 60 days.

4.8.1 Chloride content in treated water with terrestrial microalgae:

The results of chloride content in treated water with terrestrial microalgae are presented in **Table 4.13**. Chloride is widely distributed as salts of calcium, sodium and potassium in water and waste water. In potable water, the salty taste produced by chloride concentrations is variable and dependent on the chemical composition of water. In current study chloride content of initial day (0th) 70, 90, 90 and 130ppm was observed in the treated water (**T₁, T₂, T₃ and T₄**) and on 60th day, change in percentage of decreasing chloride was observed in terrestrial microalgae (A, B, C, D, E, F and G) are 28.57, 71.43, 23.81, 9.53, 28.57, 66.36 and 14.29%, respectively on **T₁**. Percentages of decreasing chloride in **T₂** are 33.33, 66.67, 18.52, 22.22, 11.11, 66.67 and 18.52%, respectively, percentage of decreasing chloride in **T₃** 29.63, 62.97, 33.33, 11.11, 22.22, 66.67 and 22.22%, respectively and percentage of decreasing chloride in **T₄** 30.77, 61.54, 30.77, 12.82, 30.77, 69.23 and 15.38%, respectively. The highest percentages of decreasing chloride as found in **T₁** was seen in *Rhizoclonium sp.* (Dandiganahalli) 71.43%, **T₂** *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu) as 66.67%, **T₃** and **T₄** are *Rhizoclonium hieroglyphicum* (Kaggodu) 66.67 and 69.23%, respectively. The lowest

percentages of decreasing chloride found in T₁, T₃ and T₄ are *Klebsormidium sp.* (Ikola) as 9.53, 11.11 and 12.82%, respectively and T₂ are *Cosmarium sp.* (ACH campus) 11.11% .

Table 4.13: Chloride content in treated water with terrestrial microalgae (ppm)

T1							
SAMPLE	0th	10th	20th	30th	40th	50th	60th
A	70.00	70.00	73.33	60.00	53.33	50.00	50.00
B	70.00	70.00	60.00	50.00	30.00	20.00	20.00
C	70.00	70.00	63.33	60.00	50.00	53.33	53.33
D	70.00	70.00	70.00	60.00	60.00	63.33	63.33
E	70.00	70.33	63.33	60.00	50.00	50.00	50.00
F	70.00	70.00	60.00	50.00	43.33	23.55	23.55
G	70.00	70.33	70.00	60.00	60.00	60.00	60.00
T2							
A	90.00	90.00	80.00	80.00	70.00	60.00	60.00
B	90.00	80.00	63.33	43.33	30.00	30.00	30.00
C	90.00	90.33	80.00	70.00	70.00	73.33	73.33
D	90.00	90.00	90.00	83.33	70.00	70.00	70.00
E	90.00	90.33	80.00	80.00	83.33	80.00	80.00
F	90.00	80.00	70.00	50.00	30.00	30.00	30.00
G	90.00	90.00	90.33	80.00	80.00	73.33	73.33
T3							
A	90.00	90.00	90.00	90.00	80.00	63.33	63.33
B	90.00	90.00	80.00	60.00	43.33	33.33	33.33
C	90.00	90.00	80.00	70.00	60.00	60.00	60.00
D	90.00	90.00	90.33	80.00	83.33	80.00	80.00
E	90.00	90.00	80.00	70.00	70.00	70.00	70.00
F	90.00	90.00	70.00	53.33	40.00	30.00	30.00
G	90.00	90.00	80.00	80.00	70.00	70.00	70.00
T4							
A	130.00	130.00	120.00	113.33	100.00	90.00	90.00
B	130.00	123.33	103.33	70.00	50.00	50.00	50.00
C	130.00	130.00	120.00	100.00	103.33	90.00	90.00
D	130.00	133.33	130.00	120.00	110.00	113.33	113.33
E	130.00	130.00	120.00	110.00	100.00	90.00	90.00
F	130.00	123.33	103.33	70.00	40.00	40.00	40.00
G	130.00	130.00	130.00	120.00	113.33	110.00	110.00

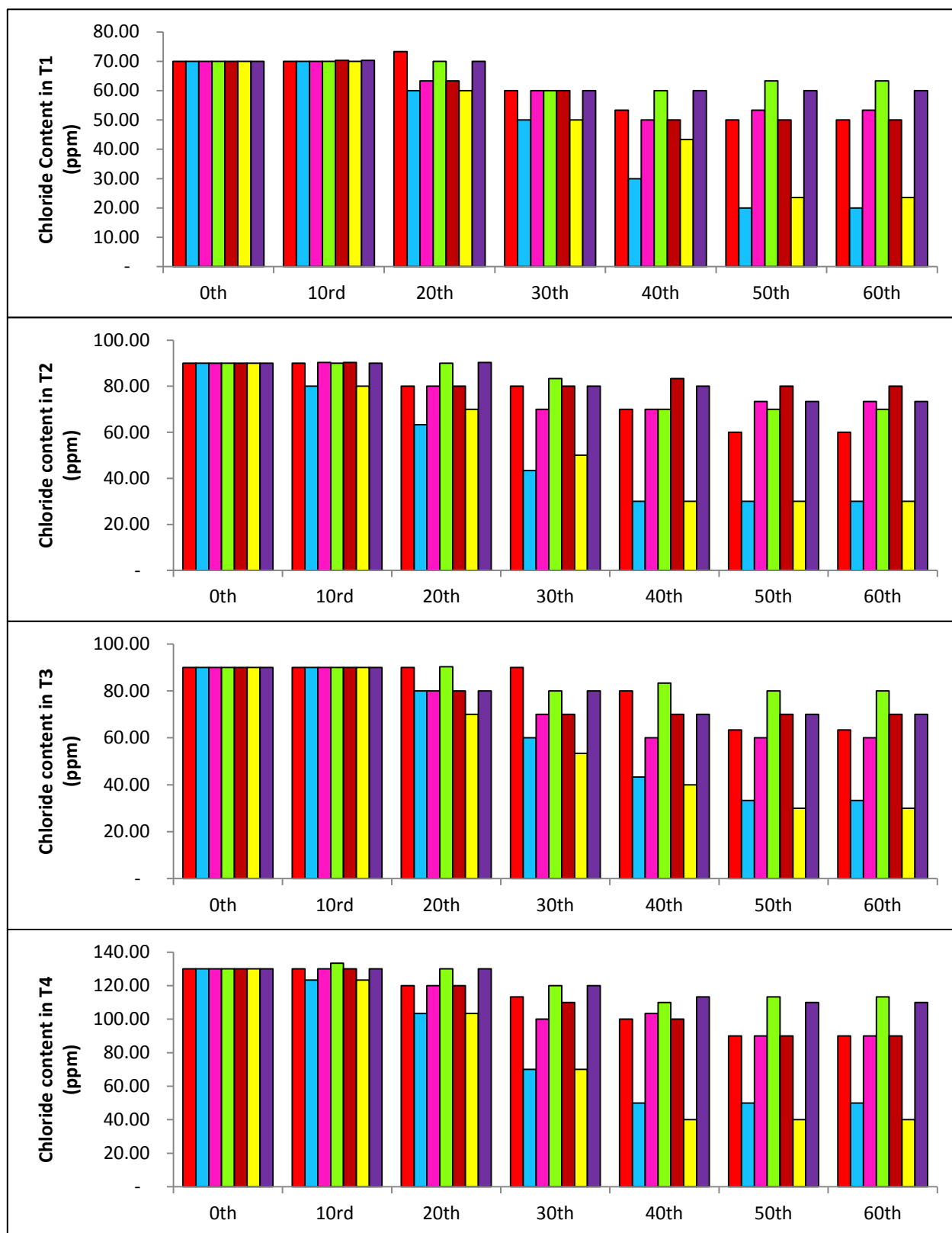


Figure 4.25: Chloride content in treated water with terrestrial microalgae (ppm)

In this present study maximum reduction of chloride content by *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu) in all type of water were observed. It is because of these two species are well adapted to environment and it can able to grow it on any type of water and these two species are resistance two stress because of high antioxidental properties. In present study *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu) were absorbing the chloride content from the water as nutrients for their growth and increased their biomass. The medium reduction of chloride content by *Spirogyra sp.* (Hoovnalli) and *Cosmarium sp.* (ACH campus) also absorbed and remaining species reducing the chloride content very less because of their growth was not good in open pond treated with different type of water. The *Rhizoclonium sp.* (Happanadka), *Klebsormidium sp.* (Ikola) and *Oedogonium sp.* (Gadanahalli) are reducing chloride very less because these three species was not grown well in open pond treated with different type of water.

4.8.2 TDS content in treated water with terrestrial microalgae:

The results of TDS content in treated water with terrestrial microalgae were presented in Table 4.14.

Table 4.14: TDS content in treated water with terrestrial microalgae (ppm)

T1								T2							
	0 th	10 th	20 th	30 th	40 th	50 th	60 th	0 th	10 th	20 th	30 th	40 th	50 th	60 th	
A	592	590	573	555	548	540	532	653	651	632	625	600	584	572	
B	586	536	501	447	373	368	345	648	586	526	459	384	376	356	
C	589	565	554	540	532	526	512	645	632	617	589	568	556	549	
D	580	543	538	530	524	511	505	656	621	617	608	591	560	552	
E	586	573	557	527	525	511	502	655	655	650	640	633	614	591	
F	590	585	528	462	389	365	349	652	638	593	518	437	419	394	
G	585	538	527	503	483	480	471	640	610	600	587	571	560	549	
T3								T4							
A	695	695	687	676	659	648	637	825	810	800	784	780	771	756	
B	680	660	613	521	443	412	396	825	823	773	694	640	624	594	
C	681	682	658	636	615	593	579	823	814	805	771	754	739	722	
D	685	674	661	645	624	613	602	820	811	802	781	768	743	731	
E	680	654	640	621	601	598	571	830	830	822	811	783	761	740	
F	682	662	627	543	470	442	418	830	829	778	706	648	621	597	
G	685	652	634	617	591	587	569	827	824	811	791	773	761	753	

In current study highest decreasing percentage of TDS was observed in *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu) it because of the absorbing salt contents in the treated water and used as nutrients for their multiplication of cell and yielding good quantity of biomass. 60th day change in percentage of decreasing nitrate content was

observed in terrestrial microalgae (A, B, C, D, E, F and G) are 10.13, 41.12, 13.07, 12.93, 14.33, 40.85 and 19.48 % respectively in T1. percentage of decreasing chloride in T2 are 12.40, 45.06, 14.88, 15.85, 9.77, 39.57 and 14.21%, respectively percentage of decreasing chloride in T3 are 8.34, 41.76, 14.97, 12.11, 16.02, 38.70 and 16.93% , respectively and percentage of decreasing chloride in T4 are 8.36, 28, 12.27, 10.85, 10.84, 28.07 and 8.94%, respectively. In all four water treatment highest reduction of TDS content was observed on two species *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* (Kaggodu).

4.9.5 pH content in treated water with terrestrial microalgae:

The results of pH content in treated water with terrestrial microalgae were presented in **Table 4.15**. In present study pH content in all four water treatment observed that the pH content in the water was gradually decreasing after 10th days in all seven present studied microalgae (A,B,C,D,E,F and G). These results indicates that all selected species are converting the alkaline water into acidic. And it was the clear indication that the selected species are well adapted to acidic water of pH 5.48- 6.4.

Table 4.15: pH content in treated water with terrestrial microalgae

T1								T2							
	0 th	10 th	20 th	30 th	40 th	50 th	60 th	0 th	10 th	20 th	30 th	40 th	50 th	60 th	
A	7.8	6.90	6.42	6.24	6.35	6.28	6.15	8.1	7.15	6.47	6.35	6.3	6.3	6.1	
B	7.8	7.10	6.45	6.0	5.71	5.6	5.48	8.2	7.68	6.95	6.68	5.95	5.8	5.7	
C	7.8	6.95	6.25	6.12	5.87	5.8	5.66	8.2	7.29	6.63	6.49	6.0	5.92	5.76	
D	7.8	6.8	5.95	5.9	5.7	5.65	5.58	8.2	7.24	6.58	6.49	6.4	6.35	6.3	
E	7.8	7.25	6.72	6.5	6.48	6.4	6.2	8.1	7.65	7.2	6.85	6.6	6.49	6.4	
F	7.8	6.55	6.01	5.98	5.62	5.6	5.53	8.1	6.8	6.35	6.01	5.58	5.5	5.5	
G	7.8	7.0	6.46	6.38	6.0	6.0	5.95	8.2	7.24	6.42	6.4	5.95	5.8	5.8	
T3								T4							
A	8.28	7.45	6.89	6.7	6.6	6.65	6.4	8.51	8.15	7.4	6.92	6.8	6.4	6.38	
B	8.24	7.61	6.99	6.5	5.96	5.9	5.76	8.38	7.46	6.86	6.48	5.85	5.85	5.74	
C	8.32	7.71	6.91	6.65	6.14	6.0	5.94	8.48	7.65	6.85	6.65	6.48	6.2	5.99	
D	8.4	7.8	6.95	6.75	6.6	6.45	6.2	8.5	7.82	6.8	6.7	6.5	6.3	6.0	
E	8.19	7.5	6.8	6.6	6.35	6.35	6.24	8.42	7.45	7.0	6.8	6.58	6.5	6.35	
F	8.29	7.25	6.45	6.12	5.54	5.42	5.4	8.46	7.46	6.65	6.2	5.86	5.8	5.7	
G	8.2	7.59	6.5	6.4	6.18	5.75	5.6	8.65	7.94	7.0	6.6	6.3	6.15	6.05	

SUMMARY AND CONCLUSION

The results of the present study are summarized as follows:

In this study, around 62 terrestrial algae were collected from the fresh water bodies of in and around of the Western Ghats regions of Karnataka. These collected algae were identified based on microscopic and biochemical characteristics of and. Out of these only 7 samples of high oil content and biomass yielding, identified on the basis of microscopic and biochemical characteristics, were used for multiplication and fermentation study. All the algae samples were identified by using compound microscope (40X and 100X) and scanning electronic microscope. These seven identified species were found to have variable lipid content due to biodiversity of species, habitat and locations, which were *Rhizoclonium sp.*(Happanadka) *Rhizoclonium sp.*(Dandiganahalli), *Spirulina sp.*(Hoovnalli) *Klebsormidium sp.*(Ikola), *Cosmarium sp.*(ACH campus), *Rhizoclonium hieroglyphicum* (Kaggodu) and *Oedogonium sp.*(Gadanahalli). Collected algae were kept for multiplication it in petri plates containing BB solid media, batch culture with BB liquid medium, and open pond system, all three methods shows the good result.

- In seven present studied terrestrial microalgae maximum growth concentration and dry weight was observed in *Rhizoclonium sp.* (2.63 ± 0.008 and 1.02 ± 0.072 g/l) collected from Dandiganahalli fallowed by *Rhizoclonium hieroglyphicum* (2.56 ± 0.150 and 0.94 ± 0.018 g/l) collected from Kaggodu, which indicates that species had highest biomass production and *Oedogonium sp.* (1.85 ± 0.005 and 0.29 ± 0.006 g/l) collected from Gadanahalli has lowest concentration of growth in batch culture method. And also *Rhizoclonium sp.* (Dandiganahalli) and *Rhizoclonium hieroglyphicum* were multiplied very fast in open pond system compared to other collected species. These two species grow very well in all four types of treated water viz, normal tap water, water with nutrient supplements, R.O. residual dispense water whose pH 8.4 and urban waste water.
- Biochemical characterization viz Chlorophyll, TSS, TRS, protein and total lipid contents was conducted on seven present studied terrestrial microalgae, in that maximum chlorophyll a, b, c and total chlorophyll (14.82 ± 0.039 , 12.42 ± 0.007 , 7.11 ± 0.002 and 13.85 ± 0.087 mg/g tissue, respectively) found in *Rhizoclonium hieroglyphicum*. The minimum chlorophyll a, b and total chlorophyll (3.31 ± 0.002 , 3.55 ± 0.151 and 3.78 ± 0.011 mg/g tissue) in *Rhizoclonium sp.* (Dandiganahalli) and lowest chlorophyll c (0.93 ± 0.014 mg/g tissue) observed in *Rhizoclonium sp.* (Happanadka).
- In current study maximum percentage of TSS obtained in the *Rhizoclonium sp.* ($44.81 \pm 0.16\%$) collected from the river of Happanadka and minimum observed in the *Klebsormidium sp.* ($34.68 \pm 0.04\%$) collected from Ikola. Out of seven terrestrial microalgae in the biochemical characterization of current study *Cosmarium sp.* ($25.30 \pm 0.60\%$) has highest percentage of TRS and *Klebsormidium sp.* ($23.80 \pm 0.92\%$) has lowest TRS content.

- In seven present studied terrestrial microalgae highest protein content was observed in *Rhizoclonium sp.* (22.24 ± 0.03 mg/g) collected from pond of Dandiganahalli and lowest observed in the *Klebsormidium sp.* (5.12 ± 0.16 mg/g) collected from Ikola. SDS-PAGE was conducted for these proteins and all current studied terrestrial microalgae bands are observed in 41kDa, 30kDa and 22kDa region in the gel it indicates the terrestrial microalgae proteins as 41kDa, 30kDa and 22kDa of molecular weight.
- Algal oil obtained from the seven present studied terrestrial microalgae was converted into biodiesel by transesterification method. In that maximum content of 80.00 ± 1.32 and 80.00 ± 1.80 % biodiesel was recovered from the *Spirogyra sp.* (Hoovnalli) and *Cosmarium sp.* (ACH campus) algal oil of 32.75 ± 1.53 and $31.50 \pm 3.12\%$, respectively.
- In seven present studied terrestrial microalgae bioethanol production was standardized based on TSS, TRS, protein, pH, microbial load, and ethanol percentage. On 21st day of fermentation *Rhizoclonium sp.* (Dandiganahalli) has decreased total soluble sugar to $13.53 \pm 1.25\%$, giving 62.43 % of maximum decreasing difference. The maximum (38.52%) reducing sugar decreased percentage difference was observed in *Oedogonium sp.* In present study protein content of *Klebsormidium sp.* was gradually increased and on 21st day *Klebsormidium sp.* show increased percentage difference of 46.37%. The rate of ethanol production maximum at pH 6.79 was observed. The microbial load of samples on 21st days increases because of yeast (*Saccharomyces cerevisiae*) utilized these sugar content so total soluble sugar content was decreased.
- Defatted algal residues were the byproducts obtained during the extraction of oil from the algae and which was used as a substrate for bioethanol production by fermentation. The highest alcohol percentage was found on *Rhizoclonium hieroglyphicum* (61.76 ± 1.12) followed by *Rhizoclonium sp.* ($59.64 \pm 0.79\%$) collected from Dandiganahalli.

On the basis of above investigation it can be concluded that the biodiesel from terrestrial microalgae is technically feasible in this experiment, 75% biodiesel with 20% glycerin was recovered from 43% algal oil. It can easily culture in open pond method for biomass production. By-products of algal oil extracts such as defatted algal residues can use as a substrate for bioethanol production. And can able to produce 62% of ethanol from it. Algae grown in waste water showed good response and it was found that it reduces the pH of alkaline water and also reduces chloride and TDS content of waste water by algae and can used for bioremediation purpose. Current studied terrestrial microalgae as good antioxidental properties so it can able to survival in stress. Based on study conducted, it can be suggested that terrestrial microalgae can be used for biodiesel and bioethanol production by growing in polluted or waste water.

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APPENDIX

Bold basal medium stock preparation:

Sl.No.	Stock solution	g/l
01	NaNO ₃	25.0
02	CaCl ₂ .2H ₂ O	2.5
03	MgSO ₄ .7H ₂ O	7.5
04	K ₂ HPO ₄	7.5
05	KH ₂ PO ₄	17.5
06	NaCl	2.5
07	EDTA	50.0
	KOH	31.0
08	FeSO ₄ .7H ₂ O	4.98
	H ₂ SO ₄	1.0ml
09	H ₃ BO ₃	11.42
10	Micronutrients	g/l
	ZnSO ₄ .7H ₂ O	8.82
	MnCl ₂ .4H ₂ O	1.44
	MoO ₃	0.71
	CuSO ₄ .5H ₂ O	1.57
	Co(NO ₃) ₂ .6H ₂ O	0.49

Dissolve each of the above substances for the trace metal solution separately, prior to adding the next on the list. Adjust the pH of the medium to 6.8 with NaOH or HCL and autoclave. BB medium was prepared by dissolving 10ml of each stock solution (1-6) in 900ml of water one by one. After proper mixing added 1ml of each stock solution (7-10) and adjusted the pH to 6.8. Final volume of the media made up to 1000ml by adding distilled water; media was autoclaved under 121°C for 15min at 15Psi.

Preparation of potassium phosphate buffer (0.1M, 7.4pH):

- Solution A: KOH (0.2M) solution = 11.22g of KOH dissolved in distilled water and made up to 1000ml.
- Solution B: KH₂PO₄ (0.2M) solution = 27.22g of KH₂PO₄ dissolved in distilled water and made up to 1000ml.
- Potassium phosphate buffer (0.1M, 7.4pH) was prepared by mixing 40ml of solution A and 50ml of solution B and diluted to 100ml with adding distilled water.

Preparation of standard NaOH solution (0.1M/ 0.1N):

- Approx. 0.1N NaOH solution: Prepared a saturated solution of sodium hydroxide as follows. Added small portions about 100g of AR grade NaOH to 100ml of water in a flask, stopper and allowed to stand until the Na_2CO_3 settles to the bottom. The clear solution which is carbonate free contains about 50g of NaOH/100ml (approx. 50% solution or 12.5M). From this solution, pipetted out 5.3ml of clear supernatant liquid and transfer to 1 liter volumetric flask and diluted up to the mark.
- Standard HCl solution (0.1N): it standardized using Na_2CO_3 .
- Phenolphthalein solution: Dissolved 0.5g of phenolphthalein in 100ml of 95% ethanol.

Method:

Using a pipette transferred 20ml of standard HCl solution into 150ml glass beaker. Added 25ml distilled water and one drop of phenolphthalein indicator. Titrated with the NaOH (approx. 0.1N) until a faint pink colour was obtained. Repeated titration 3 times and calculated the exact normality of NaOH using the relationship $N_1V_1 = N_2V_2$.

SDS – PAGE staining and destaining solution preparation:

- Mix 100ml of methanol, 80ml of distilled water, 10ml of glacial acetic acid in a beaker and added 0.5gram of coomassie brilliant blue R- 250 to beaker contain those mixing solution.
- Destaining solution was prepared by well mixing 100ml of methanol, 80ml of distilled water and 10ml of glacial acetic acid in a glass beaker.

Native - PAGE recipes for enzymes:

Particles	Gel Resolving (30ml)	Staking Gel (10ml)
Water	11.9ml	3.4ml
30% acrylamide mix	10ml	0.83ml
Tris buffer (1.5M, pH8.8)	7.5ml	-
Tris buffer pH(1.0M, 6.8)	-	0.63ml
10%APS	0.3ml	0.05ml
TEMED	0.012ml	0.005ml

Preparation of sodium acetate buffer (0.2M, pH 5.0):

- Solution A: Acetic acid (0.2M) solution = Diluted 11.55ml of acetic acid to 1000ml with distilled water.
- Solution B: Sodium acetate (0.2M) solution = Dissolved 22.22g of Sodium acetate in distilled water and made up to 1000ml.
- Sodium acetate buffer (0.2M) solution was prepared by mixing 30ml of solution A and 70ml of solution B.